

COVALENT BINDING OF FOREIGN COMPOUNDS TO PROTEINS

by

Håkan Wallin

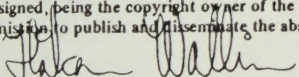


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Abstract Aspects of the covalent binding of metabolically activated foreign compounds to proteins have been investigated. The covalent binding of metabolically activated benzo(a)pyrene and phenol occurred to a limited set of the microsomal proteins of the rat liver. Cytochrome P-450 isoenzymes, microsomal epoxide hydrolase and microsomal glutathione transferase were identified as major protein targets for covalent binding benzo(a)pyrene or phenol. The metabolic activation of phenol to protein binding metabolites was catalysed by a cytochrome P-450 enzyme(s). However, the major cytochrome P-450 isoenzymes induced by -naphthoflavone or phenobarbital were quite ineffective in catalysing this reaction in a reconstituted system of isolated microsomal enzymes. The reactive metabolites which bind to protein were found to be oxidised products of hydroquinone and catechol which were the principal metabolites of phenol. The microsomal glutathione transferase was activated during metabolism of phenol, and by metabolites of phenol. It was suggested that the activation is a result of covalent modification of the enzyme. A new method, for determination of radioactive foreign compounds to proteins has been developed. The method is based on precipitation of the protein on filter paper discs, which are washed in organic solvents to remove unbound material before liquid scintillation counting. The method is rapid, simple and sensitive. An immunological method for measuring nonradioactive benzo(a)pyrene adducts to proteins, RNA and DNA was developed. The monoclonal antibody used in the assay was raised through <i>in vitro</i> immunisation. It was possible to measure small amounts of covalently bound benzo(a)pyrene with the assay, and it will be tested whether the method is suitable for monitoring environmental exposure to benzo(a)pyrene.		
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Fil. kand. Håkan Wallin (Mlm)

AKADEMISK AVHANDLING

som för avläggande av filosofie doktors examen vid matematisk-naturvetenskapliga fakulteten vid Universitetet i Lund kommer att offentligen försvaras på Kemicentrum sal B, Sölvegatan 39, Lund, torsdagen den 6 juni 1985, kl. 13.

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LIST OF SEPARATE PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their Roman numerals.

- I Wallin H., Schelin C., Tunek A. and Jergil B. (1981) A rapid and sensitive method for determination of covalent binding of benzo(a)pyrene to proteins. **Chem. -Biol. Interactions** 38, 109-118.
- II Schelin C., Wallin H., Halpert J. and Jergil B. (1984) Covalent binding of benzo(a)pyrene to cytochrome P-450 BNF-B2 and other proteins in reconstituted mixed function oxidase systems. **Chem. -Biol. Interactions** 49, 269-281.
- III Wallin H., Melin P., Schelin C. and Jergil B. The metabolic activation of phenol to species binding covalently to proteins, and phenol metabolite formation in rat liver microsomes. **Submitted.**
- IV Wallin H, and Morgenstern R. Activation of rat microsomal glutathione transeferase by metabolites of phenol **in vitro. In manuscript.**
- V Wallin H., Borrebaeck C.A.K., Glad C., Mattiasson B. and Jergil. B. (1984) Enzyme immunoassay of benzo(a)pyrene conjugated to DNA, RNA and proteins using a monoclonal antibody. **Cancer Letters** 22, 163-170.

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INTRODUCTION

Foreign compounds, or xenobiotics, are compounds that are not naturally occurring in living systems. This term is usually used in connection with hazardous chemicals and drugs. Covalent binding of foreign compounds to proteins was first observed by Miller and Miller (1947). They found that the carcinogenic dye 4-dimethylaminoazobenzene fed to rats, became covalently bound to liver proteins. This observation was the beginning of an era of investigations which led to formulation of several important concepts in carcinogenesis and toxicology. Today, it is recognized that an initial event in the development of cancer is the covalent modification of a critical cellular target by chemical carcinogens.

As most chemical carcinogens are relatively inert substances, an important finding was that these are transformed enzymatically to electrophilic metabolites which react with nucleophilic groups in cellular macromolecules (Miller and Miller, 1966). Cytochrome P-450 and related enzymes play a crucial role in these transformations. The cellular target that has received most attention is DNA, since covalent binding to DNA may result in mutation (Miller, 1978). While mutation is the most likely initial event in carcinogenesis, it can not be excluded that covalent binding to proteins is also important. Expression and duplication of the genetic message is dependent on interactions between proteins and DNA. Thus, on basis of nine points, Gronow (1980) stated that "alteration in DNA:protein complexes is an equally likely mechanism by which carcinogenesis can be induced". Proteins which have been suggested as critical targets are repressors or derepressors of the genetic message (Pitot and Heidelberger, 1963), RNA polymerases (Gronow, 1980) and histones (Jenson et al., 1982b).

Covalent binding of foreign compounds to proteins has also been implicated in their toxicity. The evidence for this is largely circumstantial, and is based on the correlation between the extent of binding and the severity of toxic effects. Early studies implicating covalent binding of foreign compounds to proteins in liver necrosis, were preformed by Brodie et al. (1971). They found that bromobenzene and other halobenzenes became covalently bound at sites of necrosis and that the extent of binding and necrosis correlated to the rate of metabolism of the compounds. Since then covalent binding of other compounds to proteins has been implicated in toxicity in various tissues, for instance, paracetamol, furesamide and carbon tetrachloride in hepatic necrosis (Gillette, 1974), 4-ipomeanol (Boyd et al., 1980), halobenzenes, paraquat and other substances in pulmonary

necrosis (Gillette, 1974), haloalkanes, acetaminophene and other substances in renal toxicity (Rush et al., 1984), and chloramphenicol (Gillette, 1974) and benzene (Snyder et al., 1981) in bone marrow toxicity. In spite of a few exceptions in the general correlation (Farber and Gerson 1984, Streeter et al., 1984a), covalent binding of foreign compounds to tissue proteins has become a widely accepted principle in toxicology. Covalent binding to proteins is frequently used to indicate toxicity in the study of the metabolism of toxins, either to explain the mechanism of action of a known toxin, or to predict the activity of a suspected one.

A number foreign compounds which have been reported to bind to proteins are listed in Table 1. Many of these are therapeutic drugs while others are environmentally distributed toxins implicated in the generation of cancer or toxic reactions. Most of the compounds require metabolic transformation in order to bind to macromolecules.

As data accumulate it becomes increasingly important to demonstrate mechanisms by which the living cell is affected through covalent binding of foreign compounds to proteins. One important finding in this context is that this binding is nonrandom. Thus, particular proteins appear to carry a predominant part of the covalent adducts. For instance, in liver cytosol and microsomes drug-metabolizing enzymes appear to be major targets for covalent modification by foreign compounds. Covalent binding to these enzymes explains the impairment in drug metabolism seen under influence of several foreign compounds, occurring both in **in vitro** and **in vivo** (cf. below).

Various aspects of covalent binding of foreign compounds to proteins are presented in this thesis. Features of the metabolic activation of phenol to protein-binding metabolites have been elucidated. Individual protein targets for binding of reactive metabolites of phenol and benzo(a)pyrene have been identified. It has been demonstrated for the first time that reactive metabolites of foreign compounds can stimulate the activity of a drug-metabolizing enzyme. Methodology for measuring covalent binding of foreign compounds has been developed.

METABOLISM OF FOREIGN COMPOUNDS

Probably all foreign compounds discussed here are subjected to enzyme-catalyzed chemical modifications after entering the organism. Generally, foreign compounds are transformed to more water-soluble derivatives which are more easily excreted, either through urine or feces. The principal chemical modifications are oxidation, reduction, hydro-

Table 1. Covalent binding of foreign compounds to proteins.

Compound	Species	Fraction ^a	Reference
Acetaldehyde	Rat	Microsomal	Nomura and Lieber 1981
Aldehydes	Rat	Microsomal	Benedetti et al, 1982
2-Acetylaminofluorene	Rat	Soluble	Miller and Miller 1952
--	--	--	Weisburger et al. 1952
--	--	Nuclear	Barry et al. 1968
--	Mouse	Microsomal	Potter et al. 1973a
--	Human	Microsomal	Dybing et al. 1979a
--	Rat	Cytosolic	Blackburn et al. 1982
Acrylonitrile	Rat	Microsomal	Peter and Bolt 1981
--	--	Hepatocytes	Geiger et al. 1983
Aflatoxins	Rat	Soluble (in L,K,S,SI) ^a	Lijinsky et al. 1970
--	--	Micosomal	Gurtoo 1973
--	--	Nuclear	Vaught et al. 1977
2-Aminofluorene	Rat	Microsomal	Hultin 1959
Amphetamine	Rabbit	Microsomal	Uehleke 1973
Aniline	Rat	Microsomal	Hultin 1959
Antipyrine	Rabbit	Microsomal	Uehleke 1973
Benzene	Rat	Microsomal	Tunek et al 1978
Benzo(a)pyrene	Mouse	Skin proteins	Miller 1951
--	--	Soluble/Partic.	Heidelberger and Moldenhauer 1956
--	Rabbit	Microsomal	Uehleke 1973
--	Rat	--	Tunek et al. 1979
--	--	Nuclear	Vaught and Bresnick 1976
--	--	--	Pezzuto et al. 1976
--	--	Cytosolic	Reeve et al. 1981
--	--	--	Schelin et al. 1983
Bromobenzene	Rat	Microsom./Cytos.	Brodie et al. 1971
Butylated hydroxytoluene	Rat	Microsomal	Nakagawa et al. 1979
CBtCl ₃	Rat	Microsomal	Sipes et al. 1973
CCl ₄	Rat	Microsomal	Sipes et al. 1973
--	Rabbit	Microsomal	Uehleke et al. 1973a
--	Rat/Mouse	Nuclear	Diaz-Gomez and Castro 1980a
CHCl ₃	Rabbit	Microsomal	Uehleke 1973
--	Rat/Mouse	Nuclear	Diaz-Gomez and Castro 1980b
Chloramphenicol	Rat	Microsomal	Pohl and Krishna 1978
Chlorobenzene	Rabbit	Microsomal	Uehleke 1973
--	Rat	Microsomal	Selander et al. 1975
Chlorpromazine	Rabbit	Microsomal	Uehleke 1973
Cyclophosphamide	Rat	Microsomal	Gurtoo et al. 1978
Cytembena	Rat	Microsomal	Mayo et al. 1982
2,4-Diaminoanisole	Rat	Microsomal	Dybing et al 1979b
Dibenz(a,h)anthracene	Mouse	Skin Nucl/Sol./Partic.	Wiest and Heidelberger 1953
--	--	Skin cytosolic	Tasseran et al. 1970
Dibenzo(c,g)carbazole	Mouse	Cytosolic	Valero et al. 1983
1,2-Dibromoethane	Rat/Mouse	Microsomal (L and ST) ^a	Banerjee and Van Duuren 1978
1,2-Dichloroethane	Rat/Mouse	Lung microsomal	Banerjee et al. 1980
Diethylstilbestrol	Rat	Microsomal	Krishna et al. 1973

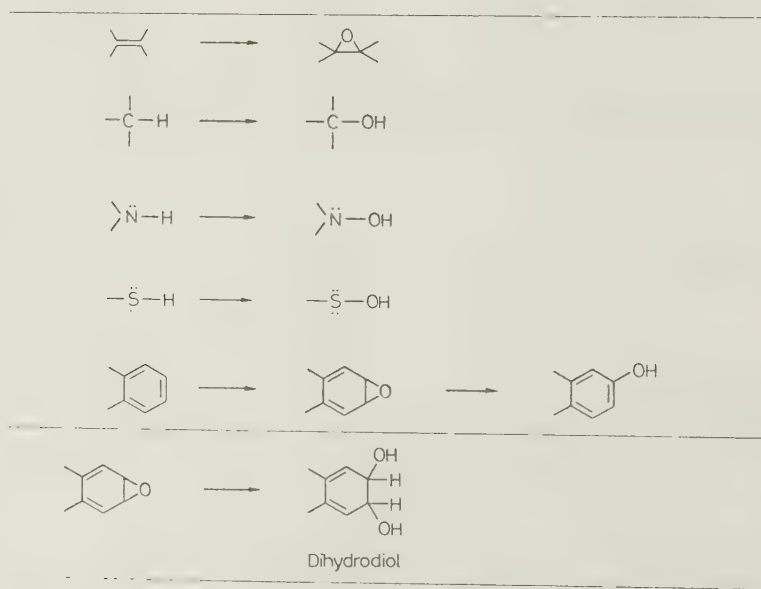
Compound	Species	Fraction ^a	Reference
Dimethylaminoazobenzene	Rat	Homogenate	Miller and Miller 1947
-"-	-"-	Soluble	Price et al. 1949
-"-	-"-	Microsomal	Hultin 1957
-"-	-"-	Cytosolic	Ketterer et al. 1967
-"-	-"-	Nuclear	Bakay et al. 1969
Dimethylbenzanthracene	Mouse	Skin sol./Particulate	Heidelberger and Moldenhauer 1956
-"-	Rat	Nuclear	Jungmann and Schweppe 1972
-"-	Rat	Adrenal Micros./Cytos.	Montelius et al. 1982
Diphenylhydantion	Rabbit	Microsomal	Uehleke 1973
Dopa	Rat	Microsomal (L/LU) ^a	McManus and Davies 1980
-"-	Rat	Microsomal	Scheulen et al. 1975
Dopamine	Rat	Microsomal	Scheulen et al. 1975
Estradiol	Rat	Homogenate	Riegel and Mueller 1954
Estrone	Rat	Homogenate	Szego 1953
-"-	-"-	Microsomal	Marks and Hecker 1969
Ethinylestradiol	Rat	Microsomal	Kappus et al. 1973
-"-	-"-	Cytosolic	Maggs et al. 1983
Ethionine	Rat	Soluble	Sorof et al. 1962
-"-	-"-	Protein in L,K,P & S ^a	Farber et al. 1967
Furesamide	Mouse	Microsomal	Potter et al. 1973b
Halothane	Rabbit	Microsomal	Uehleke et al. 1973b
-"-	Rat	Microsomal	De Groot et al. 1982
Hexachlorophene	Rabbit	Microsomal	Uehleke 1973
3'-Hydroxyacetanilide	Mouse	Microsomal	Streeter et al. 1984a
Imipramine	Rabbit	Microsomal	Uehleke 1973
4-Ipameanol	Mouse	Homogenate (L,LU & K) ^a	Jones et al. 1983
Isoniazid	Mouse	Microsomal	Thorgeirsson et al. 1973
Methylcholanthrene	Mouse	Skin sol./Particulate	Heidelberger and Moldenhauer 1956
-"-	Rat	Cytosolic	Litwack and Morey 1970
-"-	Mouse	Microsomal	Tasseron et al. 1970
-"-	Rat	-"-	Tunek et al. 1979
Naphtalene	Rat	Microsomal	Hesse and Mezger 1979
1-Naphtol	Rat	Microsomal	d'Arcy Doherty and Cohen 1984
2-Naphtylamine	Rat	Microsomal	Von der Decken and Hultin 1960
1-Nitropyrene	Rat	Proteins in LU ^a	Bond and Mauderly 1984
6-Nitrobenzo(a)pyrene	Rat	Proteins in L,K,LU & S ^a	Martin et al. 1982
Nitrosoamines	Rat	Tissue slices	Magee and Hultin 1962
-"-	Rat	Nuclear	Tuberville and Craddock 1971
-"-	Human	Erythrocytes	Kim et al. 1981
1-Octyne	Rat	Microsomal	White et al. 1984
Paracetamol	Rat	Microsomal	Potter et al. 1973
-"-	Mouse	Cytosolic	Wendel and Cikryt 1981
-"-	Rat	Hepatocytes	Devalia and McLean 1983
Parathione/Malathione	Mouse	Microsomal	Stevens 1974
PCBs	Rat	Microsomal	Shimada and Sato 1978
Chlorobiphenyl	-"-	-"-	Wyndham and Safe 1978
Dichlorobiphenyl	-"-	-"-	Hesse et al. 1978
Tetrachlorobiphenyl	Monkey	-"-	Seymour et al. 1976

Compound	Species	Fraction ^a	Reference
Phencyclidine	Rat	Homog. (Sev. Tissues)	Law 1981
Phenobarbital	Rabbit	Microsomal	Uehleke 1973
Phenol --	Rat --	Microsomal --	Tunek et al. 1979 III
Propranolol	Rat	Microsomal	Pritchard et al. 1980
Propene	Mouse	Hemoglobin	Svensson and Osterman-Golkar (1984)
Pyribenzamine	Rat	Microsomal	Rao et al. 1983
<u>trans</u> -Stilbene	Rat	Microsomal	Docks and Krishna 1969
<u>trans</u> -/ <u>cis</u> -stilbeneoxide	Rat	Microsomal	Schelin and Seidegård pers. comm.
1,1,2,2-Tetrachloroethane	Rat	Microsomal	Halpert 1982
1,2,2-Trichloroethane --	Rat/Mouse Sev. Species	Micosomal Hepatocyt. Micr L&LU ^a	Banerjee and Van Duuren 1978 Miller and Guengerich 1983
Thioacetamide	Rat	Micros./Cytos./Mitoc.	Dyroff and Neal 1981
6-Thiopurines	Sev. species	Microsomal	Hyslop and Jardine 1981
Vinylchloride/bromide	Rat	Microsomal	Guengerich et al. 1981
Urethan	Rat	Microsomal	Prodi et al. 1970

^a If not stated otherwise, subcellular fractions from liver has been examined. The abbreviations are: L, liver; LU, lung; K, kidney; P, pancreas; S, spleen; SI, small intestine and ST, stomach.

lysis and conjugation. The liver is the major site of these reactions, but also other tissues may participate significantly. For instance, kidney enzymes catalyze the formation of conjugates and blood enzymes the hydrolysis of esters and amides.

As regards nonpolar compounds oxidation is usually the initial event. During oxidative metabolism of foreign compounds many reactive metabolites are formed which can react with cellular macromolecules. Examples of reactive products that may bind to proteins are epoxides, hydroxylamines, carbonium ions and free radicals (Gorrod, 1979). Key enzymes in the oxidative metabolism of foreign compounds are mixed-function oxidases located in the endoplasmic reticulum. The mixed-function oxidase is constituted of cytochrome P-450, which catalyzes the oxidation, and NADPH-cytochrome P-450 reductase (White and Coon, 1980). Monooxygenase activity implies that an oxygen atom is inserted into carbon or heteroatomic structures. Examples of such reactions are the hydroxylations and epoxidation given in the figure.



During oxygenation of the substrate one molecule of oxygen is reduced, and NADPH is oxidized forming one molecule of water. The oxidation of NADPH is catalyzed by NADPH-cytochrome P-450 reductase, which transfers electrons to the heme iron of cytochrome P-450. The second of the two electrons needed for catalytic turnover may be derived from cyto-

chrome b₅. In addition to oxygenation mixed-function monooxygenases catalyze dealkylations, peroxidations, reductions and other reactions (Sato and Omura, 1978).

The cytochrome P-450 enzymes are a polymorph family of heme proteins varying in amino acid sequence, optical properties, and having different molecular weights of about M_r 50 000. Eight isoenzymes have been identified in the rat (Guengerich et al., 1982). The levels of individual isoenzymes are under heritable and environmental control. The concentrations of some isoenzymes increase many times by exposure of animals to foreign compounds. Induction of mixed-function oxidase enzymes usually leads to an increased rate of metabolism and elimination of the inducing agent. For instance, benzo(a)pyrene and other polyaromatic hydrocarbons induce the isoenzyme P-450_{BNF-B2} thereby increasing the oxygenation rate of the inducer. (BNF is an abbreviation of β -naphthoflavone which is commonly used as inducer of the enzyme). Phenobarbital, another common inducer, causes a proliferation of the endoplasmic reticulum and an increase of cytochrome P-450_{PB-B} and NADPH-cytochrome P-450 reductase (Snyder and Remmer, 1982).

Epoxides produced by cytochrome P-450 can be hydrolyzed by epoxide hydrolases to corresponding dihydrodiols. Of the two epoxide hydrolases that have been identified, one is present principally in the endoplasmic reticulum while the other is the cytosolic (Seideård and DePierre, 1983).

Conjugation usually is the final step in drug metabolism. During conjugation the drug is coupled to endogenous water-soluble derivatives. Important conjugates of foreign compounds are sulfate, glucuronic acid, and glutathione conjugates. The two first reactions differ from the third in that sulfatation and glucuronidation involve the reaction of nucleophilic substrates with electrophilic endogenous compounds, whereas in glutathione transfer an electrophilic foreign compound reacts with a nucleophilic thiol sulfur atom in glutathione. Sulfatation and glucuronidation are catalyzed by sulfotransferases and UDP glucuronyl transferases, respectively.

The glutathione transferases catalyze the conjugation of glutathione with lipophilic molecules containing electrophilic groups. Nucleophilic substitutions may occur at halide or epoxy groups. As with other drug-metabolizing enzymes, several isoenzymes have been identified (Chasseaud, 1978). All except one are present in the cytosol, while a distinct glutathione transferase is found in the endoplasmic reticulum and other membranes (Morgenstern and DePierre, 1985).

While conjugations usually are considered to be detoxifying reactions, reactive metabolites may be produced in some instances in these reactions. For instance, haloalkane-glutathione conjugates are labile and can be transformed to reactive metabolites. This is possibly the mechanism by which haloalkanes are toxic to the kidney (Rush et al., 1984).

ASPECTS ON THE METABOLIC ACTIVATION OF BENZO(A)PYRENE AND PHENOL TO PROTEIN BINDING BINDING METABOLITES

Some aspects on the metabolic activation of aromatic hydrocarbons to protein-binding species are presented in this thesis. Thus, metabolic activation of benzo(a)pyrene to protein-bound metabolites was several times higher in microsomes from 3-methylcholanthrene-treated rats than in microsomes from untreated rats (I). The increase in metabolic activation was due to induction of an isoenzyme, cytochrome P-450_{1A1}, while another inducible isoenzyme, cytochrome P-450_{2B1}, was quite ineffective in the metabolic activation of benzo(a)pyrene (II, Guengerich, 1982). Covalent binding was catalyzed at a K_m for benzo(a)pyrene (I) that was about half that seen for formation of polar metabolites (DePierre et al., 1975). A possible reason for this difference may be that only one metabolic turnover is required for the production of polar metabolites (DePierre, 1975), whereas protein-binding metabolites may be produced first after two or more cycles of metabolism by cytochrome P-450 (Schelin et al., 1980, Jernström, 1984b). In agreement, we have found that in our incubations the benzo(a)pyrene added can be transformed quantitatively to polar metabolites within 10 min (unpublished observation). Under the same conditions covalent binding of benzo(a)pyrene to proteins increases during more than three hours (II). Indeed, it was recently suggested that a major metabolite of benzo(a)pyrene binding to proteins is 3,7,8-trihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene. This compound formed is after three cycles of metabolic oxygenation (B. Jernström, personal communication).

Another aromatic hydrocarbon that is metabolized to protein-binding species is benzene. The epoxide of benzene has been excluded as a important protein-binding metabolite; instead covalent binding is effected after further metabolism of phenol, which is the major metabolite of benzene (Tunek et al., 1978). In paper III it is demonstrated that the metabolic activation of phenol involves transformation to hydroquinone and catechol by cytochrome P-450. However, in reconstituted mixed function oxidase systems the cytochromes

P-450_{1A1} and P-450_{1B1} were surprisingly ineffective in catalyzing this reaction. It was therefore suggested that another cytochrome P-450 isoenzyme(s) is responsible for this reaction.

The formation of hydroquinone and catechol correlated to formation protein adducts during 30 min. Nevertheless, the benzene-diols are not likely to bind to nucleophilic groups in proteins. In agreement, addition of ascorbic acid to the incubations, which reduces quinones and semiquinones to hydroquinones and catechols, resulted in a 95 % reduction of covalent binding while the transformation of phenol to hydroquinone and catechol was not affected appreciably. Probably the reactive metabolites are semiquinones, or benzoquinones, or both. Oxidation of catechols and hydroquinones can occur spontaneously or through catalysis by enzymes. For instance, microsomal fatty acid desaturases have been implicated in the oxidation of catechol (Oshino and Sato, 1971), and cytochrome P-450 has been suggested to oxidize paracetamol to a benzoquinoneimine (Streeter et al., 1984a). It is not likely, however, that cytochrome P-450 catalyzes the oxidation of hydroquinone or catechol, since covalent binding of the benzene-diols was reduced rather than increased upon addition of NADPH (III).

Another microsomal enzyme, DT-diaphorase, catalyzes a two electron reduction of quinones and is, thus, a detoxification enzyme (Lind et al., 1982). In a recent report Smart and Zannoni (1984) found that addition of DT-diaphorase to microsomal incubations decreased the covalent binding of phenol. In contradiction to this, we have found that inhibition of the microsomal DT-diaphorase by dicoumarol does not increase the covalent binding of phenol; instead binding decreased by 30 % (unpublished observations).

PROTEIN TARGETS FOR COVALENT BINDING OF FOREIGN COMPOUNDS

Covalent binding of foreign compounds appears to occur specifically to a limited set of proteins rather than randomly (cf. below).

Thus, the cytosolic protein-targets for covalent binding may vary with different foreign compounds in different tissues (Ketterer, 1980). However, major targets for carcinogens and other foreign compounds are glutathione transferases. These enzymes have been shown to bind covalently the carcinogenic compounds 4-dimethylaminoazobenzene (Ketterer et al., 1980), 3-methylcholanthrene (Singer and Litwack, 1971), benzo-(a)pyrene (Schelin et al., 1983, Dixit et al., 1982, Reeve et al., 1981) and 5,9-dimethyldibenzo(c,g)carbazole (Valero et al., 1983).

Metabolically activated benzo(a)pyrene binds preferentially to a limited set of proteins in microsomes (Tunek et al., 1979). Upon sodium dodecylsulfate polyacrylamid gel electrophoresis and fluorography, strong labelling by ^{14}C -benzo(a)pyrene metabolites was seen in a few proteins in the 50 000 M_r region. One of these proteins was induced by treatment of rats with 3-methylcholanthrene. Labelling was also seen in proteins of M_r 68 000 and 72 000 (Tunek et al., 1979). From paper II it can be inferred that major microsomal targets for covalent binding of benzo(a)pyrene are epoxide hydrolase and cytochrome P-450 isoenzymes. One interesting finding was that cytochrome P-450_{PB-B2} did not form benzo(a)pyrene adducts unless cytochrome P-450_{2NF-B2} was also present in the reconstituted system. The possible reason for this is discussed below.

Several other compounds, including 3-methylcholanthrene, benzene, phenol, chlorobenzene (Tunek et al., 1979), 7,12-dimethylbenzanthracene (Montelius et al., 1982), 2-acetylaminofluorene (Kaderbhai et al., 1981), hexachlorophene (Miller et al., 1977) and carbon tetrachloride (Frank et al., 1982) preferentially bind to proteins in the 50 000 M_r region. Phenol, chlorobenzene, and benzene will also bind covalently to a protein of M_r 72 000 (Tunek et al., 1979), whereas 2-acetylaminofluorene (Kaderbhai et al., 1981), carbon tetrachloride (Frank et al., 1982) and phenol (paper IV) will bind to a protein of about M_r 20 000. It is likely that this protein, migrating fastest among microsomal proteins upon electrophoresis, represents the microsomal glutathione transferase. In paper IV is reported that this enzyme binds metabolically activated phenol and the isolated phenol metabolites catechol and hydroquinone covalently.

In reconstituted enzyme systems cytochrome P-450 has been found to be a target for reactive metabolites formed from cyclophosphamide (Marinello et al., 1984), polychlorinated biphenyls (Shimada et al., 1981), chloramphenicol and parathione (Neal et al., 1983), and *trans*-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene (Gozukara et al., 1980).

Covalent binding of chemical carcinogens to nuclear proteins has also been observed. Generally, covalent binding is greater to nonhistone proteins than to histones which, in turn, is greater than the binding to DNA (Gronow, 1980). Covalent binding to histones has attracted particular interest. In cultured mouse and hamster embryo cells benzo(a)pyrene metabolites bound covalently to histone H1, H2A and H3 (Sculley and Zytkevicz, 1983, MacLeod et al., 1980). However, the latter authors later argued that another protein with a similar electrophoretic mobility as histone H1 rather than the histone became

arylated by benzo(a)pyrene (MacLeod et al., 1982). 2-Acetylaminofluorene has been reported to bind 2-times more to arginine-rich histones H3 and H4 than to lysine-rich histones after injection of the carcinogen to rats (Barry et al., 1968). The metabolite N-hydroxy-2-acetylaminofluorene and 4-dimethylaminoazobenzene bound preferentially to histone H2A and H4, whereas administration of 7,12-dimethylbenz(a)anthracene led to preferential labelling of histone H1 and H2B (Jungmann and Schweppe, 1972).

FACTORS DETERMINING TARGET SPECIFICITY

Thus, there is ample evidence for target specificity in the binding of various foreign compounds to cellular proteins under various experimental conditions. One exception is the random distribution of covalent adducts of ranodizole in rat liver microsomal and cytosolic proteins reported by West et al., (1982). The uneven distribution of adducts may have several explanations. First, if reactive metabolites have short half lives in comparison to their rates of diffusion, they will primarily react with target sites close to their sites of formation (Tunek et al., 1979, Frank et al., 1982). For reactive metabolites formed during cytochrome P-450 catalyzed reactions the closest site is the active site of cytochrome P-450. Indeed, covalent binding of foreign compounds to the enzyme with resulting inactivation has been observed, as will be discussed below. Also, if reactive metabolites can diffuse from their sites of formation they may be unevenly distributed between different environments. For instance, reactive metabolites may be lipid soluble, and, thus, primarily distributed in membranes where they may attack integral membrane proteins (Shimada and Sato, 1980). Other environments where reactive metabolites may be accumulated are the active sites of drug-metabolizing enzymes. This may explain why glutathione transferases and microsomal epoxide hydrolase are targets for covalent binding. Finally, it is possible that all proteins which have accessible reactive amino acid residues, such as free sulfhydryl groups, will be attacked (Shimada and Sawabe, 1983). It is likely that the specific protein-labelling patterns in the examples above are determined to various degrees by the different explanations suggested. For instance, the binding specificity of carbon tetrachloride metabolites to only two protein components in microsomes (Frank et. al 1982) is probably largely due to the reaction of metabolites before they are able to diffuse from their sites of formation. In the case of benzo(a)pyrene, both this explanation and substrate interactions between reactive intermediates and substrate-binding site of drug-metabolizing enzymes may be of importance. Substrate interactions would then account for the specific labelling of

epoxide hydrolase (II'), cytochrome P-450_{PB-B2} (II) and glutathione transferase B (Schelin et al., 1983).

It was recently shown that one of the major protein-binding species of benzo(a)pyrene was formed after three cycles of metabolism (Jernström et al., 1984b). While cytochrome P-450_{PB-B2} does not effectively catalyze the formation of protein-binding species of benzo(a)pyrene, it is possible that this isoenzyme is more effective in the formation of protein-binding species from metabolites formed by cytochrome P-450_{NF-B2}. Such secondary metabolites may then bind to cytochrome P-450 isoenzymes, perhaps at the active site. Covalent binding to glutathione transferases is probably a reflection of the association of reactive intermediates, which are substrates for these enzymes, with the lipophilic active sites. It has been shown that lipophilic sulfhydryl reagents have access to a cysteinyl residue in the active site of glutathione transferase B (Carne et al., 1979).

A prerequisite for the covalent binding is that nucleophilic amino acid residues are accessible for the reactive intermediates. The importance of this factor was illustrated in a study by Shimada and Sawabe (1984). They showed that covalent binding of reactive metabolites of a chlorinated biphenyl to added proteins correlated to the number of free sulfhydryl groups in the proteins. Another complicating factor is that different reactive intermediates seem to react with different nucleophilic groups in proteins. For instance dimethylnitrosamine **in vivo** methylates lysyl, histidyl, and cysteinyl residues (Craddock, 1965), whereas ethylene- and propylene-oxide alkylate the sulfhydryl group of cysteine, the N-3 position of histidine, and other amino acids in hemoglobin (Farmer et al., 1982, Jensen et al., 1984). The liver carcinogen 4-dimethylaminoazobenzene **in vivo** reacts with methionyl, tyrosyl and cysteinyl residues (Ketterer and Christodoulides, 1969, Miller and Miller 1966). The carcinogen benzo(a)pyrene reacts with the lysine residues of histone H1 (Kurokawa and Sugano 1983, Jenson et al., 1982a), while the antibiotics ronadizole and chloramphenicol have been shown to bind to cysteinyl and lysyl residues, respectively (Wislocki et al., 1984, Halpert, 1981). During the metabolic activation of the insecticide parathione, atomic sulfur is released which binds to cysteinyl residues (Neal et al. 1981).

It is unknown, so far, why different electrophilic metabolites show different specificities for nucleophilic amino acid groups. Presumably, inherent factors of the reactants such as electric charge, steric factors, etc., are important. For instance, it may be suggested that "hard" electrophiles would react preferentially with amino groups

while "soft" electrophiles would prefer cysteinyl residues. It should be pointed out, however, that rather little is yet known on the target specificity, and that the observed differences, therefore, largely may be due to analytical difficulties, or may even be artefacts.

EFFECTS OF COVALENT BINDING OF FOREIGN COMPOUNDS ON ENZYMES AND STRUCTURAL PROTEINS

Covalent binding of foreign compounds to proteins may cause structural changes which result in altered catalytic or other functional properties. For instance, enzymes may be inactivated, or interactions between macromolecules may be disturbed. In paper IV is suggested that an enzyme (microsomal glutathione transferase) is stimulated by covalent modification. Irreversible inactivation by products formed during catalysis is often caused by covalent binding of reactive intermediates. This type of inactivation, termed suicide inactivation or mechanism-based enzyme inactivation, has been used for designing drugs specifically acting at target enzymes (Rando, 1984). The many aspects of covalent modification can not be covered here. Therefore, the discussion will be restricted to effects on drug-metabolizing enzymes and some proteins that may be important in toxic reactions.

EFFECTS ON THE MIXED-FUNCTION MONOOXYGENASE SYSTEM

Because reactive intermediates are formed during cytochrome P-450-dependent metabolism of many lipophilic compounds, these may become trapped within the active site of the enzyme. Resulting suicide inactivation of cytochrome P-450 has been seen in clinical practice as well as in animal studies manifested in decreased mixed-function oxidase activity, retarded drug elimination, prolonged drug duration and conversion of hepatic heme to abnormal green pigments (De Matteis, 1981). **In vitro** inactivation of cytochrome P-450 requires molecular oxygen and NADPH (Ortiz de Montellano and Correia, 1983). An exception is the inactivation caused by carbon tetrachloride and halothane, which is dependent on reductive metabolism (cf. below).

Well-known suicide inactivators of cytochrome P-450 are molecules containing allyl-, vinyl or ethynyl groups (DeMatteis, 1981, Ortiz de Montellano and Correia, 1983). Among these molecules are therapeutic drugs like secobarbital (Levin et al., 1972) 2,2-diethyl-4-pentenamide (Brinshulte-Freitas and Uehleke, 1979), ethynyl sterols such as norgestrol (Ortiz de Montellano et al., 1979), and danazol (White, 1978). These molecules are metabolically activated to reactive intermediates, which probably react with the nitrogen of the pyrrole ring in heme,

resulting in a loss of heme iron. The reactive intermediates are most likely radicals with unpaired electrons (Ortiz de Montellano and Correia, 1983). Recent work has indicated a similar mechanism for the inactivation of cytochrome P-450 by some nitrogen-containing compounds, such as hydrazines (Muakkassah and Yang, 1981, Jonen et al., 1982) and 3,5-bis-carbethoxy-2,4,6-trimethyl-1,4-dihydropyridine (Tep-hly et al., 1979).

Acrolein a highly reactive allylic aldehyde, was recently reported to cause total destruction of NADPH-cytochrome P-450 reductase in lung and liver microsomes, presumably due to covalent modification of the enzyme (Patel et al., 1984).

Covalent binding of sulfur to cytochrome P-450 has been reported during oxidative desulfuration of parathione (Neal et al., 1983) and carbon disulfide (Dalvi et al., 1974, De Matteis 1981). The covalent binding is accompanied by inactivation of cytochrome P-450, but does not cause destruction of heme. The major part of the incorporated ^{35}S from parathione could be released readily upon treatment with dithio-threitol or cyanide, suggesting that the ^{35}S is bound to cysteine in hydrodisulfide linkages (Neal et al., 1983). The reactive inter-mediate of these compounds are probably singlet atomic sulfur (Neal et al., 1983, De Matteis, 1981).

Several other sulfur-containing chemicals have been reported to inhibit drug oxidation or to destroy cytochrome P-450. These include compounds like disulfuram (antabuse), diethyldithiocarbamate and bis-(ethylxantogen) (Hunter and Neal, 1975), organophosphate insecticides with P=S groups (Uchiyawa et al., 1975) and the lung toxin -naphthyl thiourea (Boyd and Neal, 1976). Oxidative desulfuration has been implicated for all these substances.

The antibiotic chloramphenicol is a suicide substrate of the major phenobarbital-induced form of cytochrome P-450 (Halpert and Neal, 1981). The reactive metabolite, most likely an oxamyl chloride, binds principally to ϵ -amino groups of lysine. *In vitro*, chloramphenicol is a very effective suicide substrate requiring approximately five turn-overs to inactivate the enzyme (Halpert, 1981).

Treatment of animals with carbon tetrachloride causes extensive morphological changes, enzyme destruction and lipid peroxidation in the liver, ultimately leading to hepatic necrosis. Reductive metabolism of carbon tetrachloride precedes the loss of cytochrome P-450 activity in the liver and in microsomes *in vitro* (De Matteis, 1981). It is

disputed, however, whether covalent binding of reactive metabolites alone is sufficient to destroy cytochrome P-450 or whether lipid peroxidation is a prerequisite (De Matteis, 1981, De Groot and Haas, 1981).

The mechanism of inactivation of cytochrome P-450 by halothane is similar to that of carbon tetrachloride. NADPH and hypoxic conditions are required, and especially cytochrome P-450 isoenzymes induced by phenobarbital were susceptible to destruction (De Groot et al., 1982)

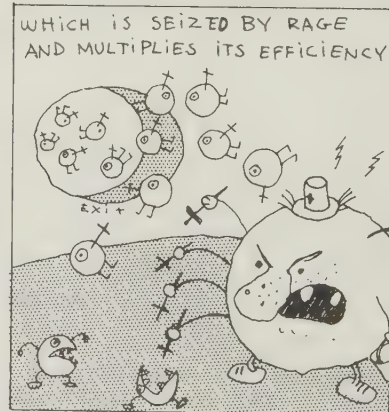
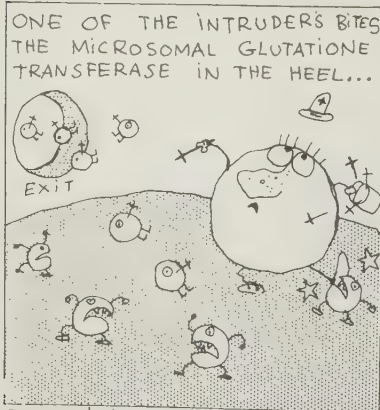
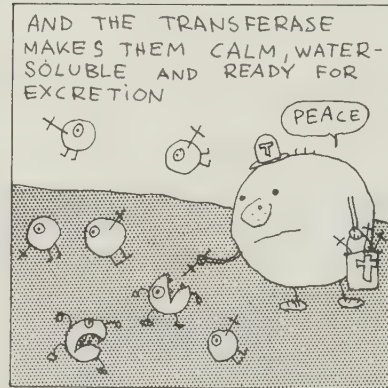
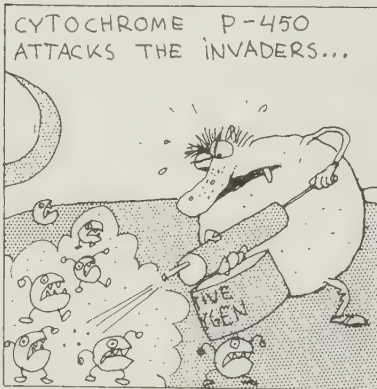
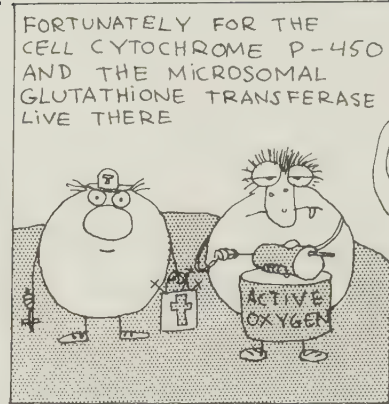
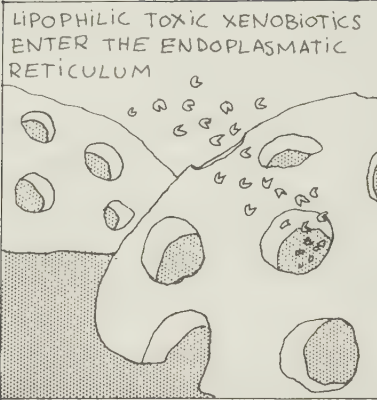
EFFECTS ON GLUTATHIONE TRANSFERASES

Carbon tetrachloride and ethylene dibromide decrease cytosolic glutathione transferase activity in livers of rats exposed to these compounds (Botti et al., 1982). From experiments with isolated cytosolic glutathione transferases it was demonstrated that these enzymes are inactivated irreversibly also *in vitro* by reactive metabolites of ethylene dibromide (Ivanetich et al., 1984).

Treatment of rats with chloroform caused a decrease in the liver glutathione transferase activity, presumably under impact of reactive metabolites (Aniya and Anders, 1985). The activity associated with the basic glutathione transferases AA, A, B, and C decreased with time during treatment. After incubation of partly purified enzymes with chloroform in the presence of microsomes and NADPH, the activity of all transferases, except form C, was abolished. The authors suggested that phosgene, formed during oxidative metabolism of chloroform by the mixed-function oxidase, bound to amino acid residues in the enzyme thereby causing inactivation.

In contrast to the above mentioned inhibition of cytosolic glutathione transferase activities by carbon tetrachloride and ethylene dibromide, Botti et al (1982) found an increase in the activity of glutathione transferase in liver microsomes. The activity increased by 50 % 2 h after administration of the substances to rats. This fast response made it unlikely that the increase was a result of enzyme induction. The microsomal glutathione transferase has similar catalytic properties as the cytosolic enzymes, but lower molecular weight, and different immunological determinants (Morgenstern and DePierre, 1985). In addition, the microsomal enzyme can be activated up to 15-fold by sulfhydryl reagents. Such a high degree of activation, observed with isolated enzyme, required complete blockage of the single cysteine sulfhydryl group of the enzyme (Morgenstern and DePierre, 1983).

Cartoon 1.



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In paper IV is presented evidence that the microsomal glutathione transferase is activated during cytochrome P-450-dependent metabolism of phenol. Three metabolites of phenol, hydroquinone, benzoquinone and 1,2,4-benzenetriol, were more potent in activating the enzyme than phenol itself, while catechol had no significant effect. The isolated enzyme was not affected by phenol, hydroquinone, or catechol, but was stimulated up to 6-fold by benzoquinone and benzenetriol. Reactive metabolites of phenol bound covalently to the enzyme, as did hydroquinone and catechol. Maximal activation of the enzyme was reached within 20 s. After that the activity decreased to reach the basal level 10-20 min later. It is suggested that the activation was a result of covalent modification of the enzyme in analogy with the activation by sulfhydryl reagents. The following decrease may result from the instability of the enzyme in absence of reduced glutathione. It is not known whether the activation reflects a mechanism operating *in vivo*. However, it is tempting to suggest that the microsomal glutathione transferase can adapt to exposure to foreign compounds (cf. cartoon 1). Then, reactive metabolites, formed during oxidative metabolism, will bind to the enzyme. The activity of the enzyme then increases, thereby increasing the rate at which reactive metabolites are eliminated through conjugation with glutathione.

An analogy of the action of sulfhydryl reagents and metabolites of phenol and benzene has been observed before. Thus, these substances can inhibit mitosis of cells, and blastogenesis of lymphocytes in culture (Irons et al., 1981). Hydroquinone also inhibits polymerization of purified tubulin, as do sulfhydryl reagents, and becomes covalently bound to the high molecular weight fraction of tubulin (Irons and Neptun 1980). These findings may be related to the G₂/M block seen in after benzene exposure of proliferating cells in bone marrow (Muirhead et al., 1980), abnormalities in cell division (Tunek et al., 1982), and aplastic anemia and leukemia (Snyder et al., 1981).

EFFECTS ON PROTEINS WHICH INTERACT WITH DNA

In the eukaryotic cell nucleus DNA is compacted with histones to chromatin. The transition from high to less condensed chromatin is believed to be related to gene expression (Larson and Weintraub, 1982). In this context it is interesting that covalent binding of metabolically activated benzo(a)pyrene to histone H1 decreases its affinity for DNA (Jenson et al., 1982). It is possible that a decreased affinity of histones for DNA leads to unfolding of chromatin, or that binding of the bulky benzo(a)pyrene group to histones could interfere with the accurate reading of the DNA template during transcrip-

tion and replication.

Many carcinogens affect RNA synthesis (Gronow, 1980). This effect may be explained by an impaired fidelity of transcription by RNA polymerase II (Glazer 1978). It can not be concluded if this impairment, of the RNA polymerase II isolated from rats treated with N-hydroxy-acetylaminofluorene, is due to covalent modification of the enzyme. However, RNA and DNA polymerases may be inactivated by N-methyl-N-nitrourea by methylation of the sulfhydryl group of the active site (Grisham and Smith, 1984).

METHODOLOGY FOR MEASURING COVALENT BINDING OF FOREIGN COMPOUNDS TO PROTEINS

Several methods have been developed for measuring covalent binding of foreign compounds to proteins. These can be divided into two groups according to their purposes and technical features. In most investigations covalent binding has been measured after administration of radiolabelled foreign compounds to animals or after testtube incubations of cells or subcellular fractions. Differing in methodology are methods developed with the aim to determine exposure of foreign compounds to human objects. These methods require very sensitive analyses of minute amounts of non-radioactive covalent adducts. Two different approaches have been used for this purpose: gas chromatography-mass-spectrometry and immunological methods.

EXHAUSTIVE EXTRACTION METHODS

The methods developed for measuring the binding of radiolabelled (or coloured) substances will be referred to here as exhaustive extraction methods, to distinguish them from the methods designed for measuring non-radioactive adducts. These methods are based on the removal of unbound compound from those bound to proteins after exposure of animals or *in vitro* systems to radiolabelled or colored compounds. This separation is accomplished by precipitation of the protein fraction in acidic or alcoholic solvents followed by exhaustive extraction of the precipitate with organic or acidic solvents. The method developed by Jollow et al. (1972) to measure covalent binding of ^3H -acetaminophene to mouse liver proteins illustrates this technique. After administration of the compound, tissue samples were homogenized and macromolecules were precipitated with 0.6 M trichloroacetic acid. After centrifugation, the sediment was resuspended and centrifuged twice in 0.6 M trichloroacetic acid and six times in 80 % methanol, or until no

further radioactivity could be removed. The protein precipitate was dissolved in 1 M NaOH, and the radioactivity was measured by liquid scintillation counting. This method, or similar approaches, have been used by others (for example, Pohl and Branchflower, 1981, Sun and Dent, 1980, Nakagawa et al., 1979, Miller III et al., 1977). In some cases nucleic acids were removed before precipitation of the protein (for example, Kahl et al., 1977, Huberman and Sachs, 1977, Kuroki and Heidelberger, 1971), and sometimes, when polyaromatic hydrocarbons were used as substrates, the homogenate was extracted with ethyl-acetate before precipitation (for example, Montelius et al., 1982).

In our laboratory we have used the Jollow method and similar methods (I, Tunek et al., 1978). Disadvantages with these methods are that they are time consuming and laborious particularly when a large number of determinations have to be made. It is also required comparatively large amounts of material. Therefore, we examined whether precipitation in filter papers would be suitable for measuring protein binding of radiolabelled polyaromatic hydrocarbons. The results of these investigations are presented in paper I. The method was developed for measuring the covalent binding of benzo(a)pyrene to microsomal preparations. After incubation reaction mixtures were applied on filter paper discs, and the protein was precipitated by immediate immersion of the discs in ethanol. The discs were washed several times in organic solvents, dried, and the radioactivity was measured by liquid scintillation counting.

This method allowed us to carry out a large number of determinations at the same time, since the filter papers could be collectively handled in one vessel, and since resuspension and centrifugation were not required. The unbound material was efficiently removed with no measurable loss during the extraction procedure. It was possible to scale down incubation mixtures and to use dilute protein-solutions with retained accuracy in the determinations. In contrast, precipitation of protein from solution, as in the Jollow method, is incomplete, and especially low M_r proteins are precipitated inefficiently (Sun and Dent, 1980). Consequently, if a major part of the binding is associated with low M_r proteins a significant amount of the covalent binding may remain undetected. During the development of our method we found that a similar approach had been used by Uehleke et al. (1973) for measuring covalent binding of carbon tetrachloride to proteins. However, they used trichloroacetic acid for precipitation and washing of filters, which made the method unsuitable for measuring covalent binding of polyaromatic hydrocarbons and other water insoluble substances. In another report, published simultaneously with ours, Reeve

and Gallagher (1981) used a similar method for measuring covalent binding of benzo(a)pyrene in rat liver tissues.

Table II lists investigations in which our method has been used. The method has proved to be useful for measuring compounds of varying polarity in various cellular fractions. Interestingly, the method has been used also for measuring covalent binding to isolated cells. It should be noted, however, that we have found that glutathione conjugates of phenol and stilbene oxides are not easily removed by the standard washing procedure. This drawback can be overcome by adding a few washings of the discs in 10 % trichloroacetic acid.

Another technique was developed by Sun and Dent 1980. They used sodium dodecylsulfate equilibrium dialysis to separate unbound material from that bound to macromolecules. For reasons unknown the covalent binding was up to three times higher than when measured by an exhaustive extraction method.

For the majority of the methods no conclusive evidence exists that the binding measured is covalent. It is merely been assumed that the exhaustive extraction in of the appropriate solvents would release all non-covalently bound substances. The assumption originates from investigations of Miller and Miller (1966) and Jollow et al. (1972) who concluded, after thorough examination, that the bound material was covalently bound to amino acid residues. As discussed above, in some instances individual amino acid-foreign compound-adducts have been isolated and identified.

MEASUREMENT OF NON-RADIOACTIVE ADDUCTS

Methods have also been developed for determining covalent adducts in the blood of human objects exposed to foreign compounds (Ehrenberg and Osterman-Golkar 1980). The strategy in these methods is to degrade hemoglobin and quantify modified amino acids by gaschromatography-mass spectrometry (Calleman et al., 1978, Farmer et al., 1982). In recent development the, N-terminal valine of hemoglobin is released through Edman degradation of the hemoglobin (Jenson et al., 1984)

A quite different approach are the immunological methods. Immunoassays have been used for measuring a variety of small molecules and macromolecules, including carcinogens bound covalently to DNA (Müller and Rajewski, 1981, Poirier, 1981), and many toxic compounds (Despaux et al., 1976). The basis of immunoassays are antibodies specifically reacting with the molecule measured. Antibodies can be obtained through

Table II.

Compound	Species	Reference
Benzo(a)pyrene - _n - -diolepoxides -diolepoxises	Microsomal & cytolie Isolated enzymes Microsomal Isolated hepatocytes	Schelin et al. 1983 II Jernström et al. 1984a Jernström et al. 1984b
p-Bromophenol and bromocatecol	Microsomal	Wolff pers. comm.
Dimethyl benzanthracene - _n -	Microsomal Adrenal Cells	Montelius et al. 1982 Hallberg & Rydström, 1983
Phenol, catechol & hydroquinone	Microsomal	III
Steroids	Postmitochondrial supernatant	Tunek pers. comm.
cis- & trans- stilbeneoxide stilbeneoxide	Micros. & cytos.	Schelin & Seidegård pers. comm.
Trichloroethylene	Micros. & isolated hepatocytes	Miller and Guengerich, 1983

administration of an immunogen to an animal or through **in vitro** immunization of lymphocytes in culture. Since it is not possible to obtain an immuneresponse, resulting in antibody production with small molecules, these have to be coupled to proteins (or other macromolecules) before immunization.

Covalent binding of carcinogens to DNA has been analysed by immunoassays in several instances (Montesano et al., 1982, Poirier, 1981, Müller and Rajewsky, 1981) The high sensitivity and capability of measuring non-radioactive adducts suggest the use of these methods in monitoring human exposure to carcinogens. Indeed, it was recently possible to detect benzo(a)pyrene adducts in the tissues of lung cancer patients by an immunoassay, while no adducts were found in a control group (Perera et al., 1982).

Since, covalent binding of foreign compounds to proteins often is greater than binding to DNA, and proteins are more easily obtained from human tissues, it can be suggested that proteins are a better material than DNA for these analyses. Nevertheless, only two methods has been developed in the purpose of measuring protein adducts of foreign compounds (V, Santella, personal communication). Both these immunoassays were developed for measuring benzo(a)pyrene adducts, and are based on monoclonal antibodies.

The antibody described in paper V was raised through **in vitro** immunization. Thus, isolated mouse spleen cells were cultured with 6-amino-benzo(a)pyrene coupled via an amido bond to bovine serum albumin. The immunized cells were immortalized through fusion with myeloma cells and cloned according to traditional protocol for production of monoclonal antibodies. One cell line which produced high titre antibodies was further cultured to obtain a reasonable amount of antibodies. The antibody reacted with benzo(a)pyrene chemically coupled to ovalbumin, and with metabolically activated benzo(a)pyrene bound to DNA, RNA and microsomal proteins. It is likely that the sensitivity of the immuno assay must be improved if it shall be used for monitoring human exposure to benzo(a)pyrene. This can probably be achieved by using more sensitive. Also digestion of the protein into smaller fragments can make the benzo(a)pyrene adducts more accessible for reaction.

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I

A RAPID AND SENSITIVE METHOD FOR DETERMINATION OF COVALENT BINDING OF BENZO[a]PYRENE TO PROTEINS

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SUMMARY

A method is presented for the quantitative determination of covalent binding of metabolically activated benzo[a]pyrene to microsomal proteins. After incubation of radiolabelled benzo[a]pyrene with microsomes and NADPH, the mixture is applied to filter paper discs. These are immersed in ethanol to precipitate the proteins. Unbound radiolabel is removed by repeated washes of the filters in organic solvents before scintillation counting. The method is simple, rapid, sensitive and accurate, and works both with ¹⁴C- and ³H-labelled compounds. The method is suitable for measuring the incorporation of other radiolabelled xenobiotics to proteins of both microsomes and other subcellular fractions and for the analysis of binding to isolated proteins.

INTRODUCTION

Many xenobiotics are known to bind covalently to cellular macromolecules, either directly or after metabolic activation by the microsomal mixed-function oxidase system [1,2]. The covalent binding of reactive metabolites to DNA and its possible correlation to mutagenesis and carcinogenesis have been extensively investigated [3]. In comparison, the binding of metabolites to proteins has received little attention, although quantitatively this binding exceeds that to DNA and RNA in several experimental systems [4,5]. The covalent binding of reactive metabolites of xenobiotics to proteins has been implicated in cell toxicity [5–8]. In this connection the findings that metabolically activated toxic and carcinogenic compounds will bind specifically rather than randomly to microsomal [9–11] or nuclear [12,13] proteins are of considerable interest.

Abbreviations: MC, 3 methylcholanthrene; PB, phenobarbital; SDS, sodium dodecyl-sulfate.

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A serious limitation when studying the covalent binding of metabolically activated compounds to proteins is the lack of convenient quantitative analytical methods. Usually the incorporation is studied using radiolabelled substances. After the incubation unbound compounds have to be removed from the protein fraction containing radiolabelled adducts. This may be achieved through extractions with organic solvents and/or precipitation of the proteins and repeated washings of the precipitate [12–18]. Since each step involves a centrifugation the processing is rather cumbersome when several samples are to be analyzed. In addition, comparatively large incubations have to be performed with these methods in order to get an acceptable recovery of bound material. A recently described method [19] employs dialysis in the presence of SDS to remove unbound material. This method appears useful for measuring covalent binding to macromolecules in general, i.e., including nucleic acids and complex lipids, but it is not suitable for rapid analysis of the binding to proteins.

Using another method Uehleke et al. [20] have studied the covalent binding of ^{14}C -labelled carbon tetrachloride to microsomal proteins by precipitation of the proteins on filter paper discs after incubation. Trichloroacetic acid was employed for precipitation and washing of the filters. However, this technique is not suitable for analysis of the binding of polycyclic aromatic hydrocarbons since excess radiolabel can not be removed efficiently after precipitation in trichloroacetic acid.

In this report we present an alternative method for the quantitative analysis of benzo[*a*]pyrene covalently bound to proteins. It involves precipitation of proteins with bound radiolabelled benzo[*a*]pyrene metabolites on filter paper discs using organic solvents. Unbound radiolabel is then extracted with organic solvents while the proteins are still precipitated on the discs. The method is rapid, simple and sensitive, and it can easily be adapted for the analysis of other covalently bound xenobiotics. The incubation conditions for optimum binding have been studied.

MATERIALS AND METHODS

Chemicals

[^{14}C] Benzo[*a*]pyrene, [^3H] benzo[*a*]pyrene and [^{14}C] valine were obtained from the Radiochemical Centre, Amersham. The preparation of ^3H -labelled benzo[*a*]pyrene metabolites was as described earlier [21]. All chemicals were of analytical grade, except organic solvents used for washing of filters.

Preparation of microsomes

Liver microsomes were prepared from male Sprague–Dawley rats [10]. The animals had been treated with PB (0.1% in the drinking water for 5 days), MC (one i.p. injection of 80 mg/kg body wt. in corn oil 24 h before sacrifice) or were untreated (control microsomes). The specific contents of cytochrome *P*-450 determined according to Omura and Sato [22] were 1.43, 0.70 and 0.59 nmol/mg protein, respectively. Protein was determined by the Lowry method [23] with bovine serum albumin as standard.

Liver microsomes radiolabelled in the protein fraction were obtained in the following way. PB (75 mg/kg body wt.) was injected once i.p. followed after 4 and 18 h by two i.p. injections of [^{14}C]valine (10 μCi each; 285 mCi/mmol). The rats were killed 4 h after the last injection. Microsomes were prepared as above and washed twice more in buffer also containing 1 mM unlabelled valine. No free radiolabelled valine could be detected in this preparation by thin layer chromatography [24] and 99.8% of the radioactivity was recovered after precipitation with 5% trichloroacetic acid.

Analysis of the covalent binding of benzo[a]pyrene to microsomal proteins

The standard incubation procedure arrived at after optimization of the conditions was the following. The incubation mixture contained 50 mM Tris-HCl (pH 7.5), 5 mM MgCl_2 , 5 μM MnCl_2 , 2 mM NADPH, 25 μM [^{14}C]benzo[a]pyrene (21.7 mCi/mmol) dissolved in 2.5 μl dimethylsulfoxide and 100 μg microsomal protein in a final volume of 100 μl . The incubation was started by the addition of benzo[a]pyrene. It was performed in stoppered tubes at 37°C for 30 min. The incubation was stopped by transferring a 50- μl aliquot to a filter paper disc (Whatman 3MM, diameter 2.5 cm) which was immediately immersed in 96% ethanol.

To remove unbound benzo[a]pyrene and benzo[a]pyrene metabolites formed during the incubation, the discs were washed consecutively in ethanol, twice in methanol, twice in acetone and once in *n*-hexane. Each wash was for 10 min with occasional agitations in 10 ml of solvent per disc. Several discs could be washed together without cross-contamination of radioactive material. The discs were dried and counted in 0.4% Omnifluor (New England Nuclear) in toluene using a Searle Mark III liquid scintillation counter. The recovery of radioactivity was 96% for ^{14}C and 53% for ^3H when compared to combusted samples. The values given have been compensated

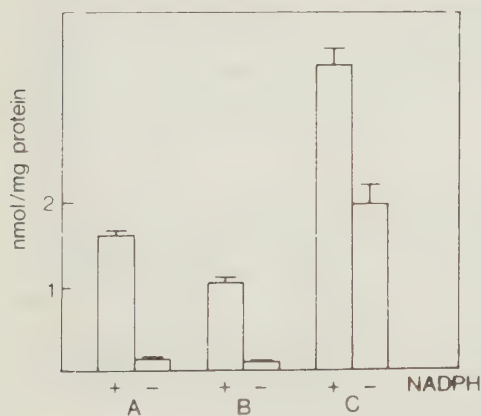


Fig. 1. A comparison between different extraction procedures. Scaled-up incubations were performed as described in Materials and Methods using microsomes (2 mg/ml) from MC-treated rats. Aliquots of 50 μl were applied on filter paper discs which were washed as described in Materials and Methods (A). Aliquots of 300 μl were extracted according to Tunek et al. [18] (B) or Jollow et al. [14] (C). Incubations without NADPH were performed as blanks. Each value represents the average of 10 determinations.

for recovery and counting efficiency. The standard deviation of the analyses performed with this method is 3.6% ($n = 10$) (cf. Fig. 1).

Analysis of the binding of benzo[a]pyrene to microsomal RNA

After incubation a scaled up standard incubation mixture (300 μ l) with microsomes from MC-treated rats was made 0.5 M in NaCl (final vol. 1 ml). The mixture was extracted twice in 1 ml of phenol previously saturated with water. Each of the phenol phases was washed with 0.5 ml of 0.5 M NaCl. The aqueous phases were combined and made 5% in SDS (final vol. 2 ml). Twelve millilitres of 95% ethanol saturated with sodium perchlorate was added and nucleic acids were allowed to precipitate overnight at -20°C . The precipitate was collected by centrifugation and washed 3 times with 70% ethanol. It was dissolved in 1 ml 0.15 M NaCl and 0.015 M sodium citrate (pH 7.2). RNA was determined with a modified orcinol method [25]. Bound benzo[a]pyrene was measured by scintillation counting in 5 ml Aquasol (New England Nuclear).

RESULTS

Precipitation of proteins and washing procedure

Two parameters are particularly important for a method to be useful in the analysis of protein-bound radiolabelled compounds. First, the recovery of radiolabelled protein adducts should be quantitative and, second, unbound material should be removed efficiently to keep the background low. Table I shows that prelabelled microsomal proteins are recovered quantitatively after precipitation on filter paper discs followed by washing according to our procedure. In contrast, earlier used methods [14,18] gave a slightly lower recovery of proteins.

The efficiency of the washing procedure was tested in experiments where radiolabelled benzo[a]pyrene or metabolites of benzo[a]pyrene were included in the incubation mixture without NADPH. Samples were applied on filters which were washed as in our method. The benzo[a]pyrene meta-

TABLE I

RECOVERY OF PROTEIN BY DIFFERENT EXTRACTION PROCEDURES

Microsomal protein (330 $\mu\text{g}/\text{sample}$) labelled with [^{14}C]valine was used. Each value represents the average of 10 measurements.

Treatment	Recovery	
	dpm	%
Before extraction	406 $\pm$ 16	100
Present method	407 $\pm$ 14	100
Tunek et al. [18]	330 $\pm$ 56	81
Jollow et al. [14]	299 $\pm$ 12	74

bolites tested (3-hydroxy, 9-hydroxy, 4,5-dihydrodiol, 7,8-dihydrodiol, 9,10-dihydrodiol and 4,5-oxide) were removed with a similar efficiency as benzo[a]pyrene itself. This indicates that metabolites formed during an incubation do not influence the background by not being removed efficiently during the washing procedure.

A comparison between different extraction methods after incubation of benzo[a]pyrene with microsomal proteins is presented in Fig. 1. Both the new procedure and the one we used earlier [18] gave low background values in the absence of NADPH. Unbound material was not extracted efficiently by a third method [14], which therefore yielded inexact results.

Incubation conditions

The time-dependent binding of benzo[a]pyrene to microsomal proteins is shown in Fig. 2. It was linear (microsomes from PB-treated or control rats) or close to linear (microsomes from MC-treated rats) for at least 60 min. The incorporation continued at a slightly slower rate after this time. At 220 min, 18, 9 and 6 nmol of metabolically activated benzo[a]pyrene or benzo[a]pyrene metabolites had bound per milligram microsomal protein from MC- or PB-treated and control rats, respectively. The incorporation in absence of NADPH was low indicating that metabolic activation was necessary for binding to occur. Despite the extensive binding to microsomes from MC-treated rats oxygen was not a limiting factor in our incubations.

The influence of the amount of microsomal protein on the incorporation of benzo[a]pyrene is seen in Fig. 3. The reaction was proportional to added

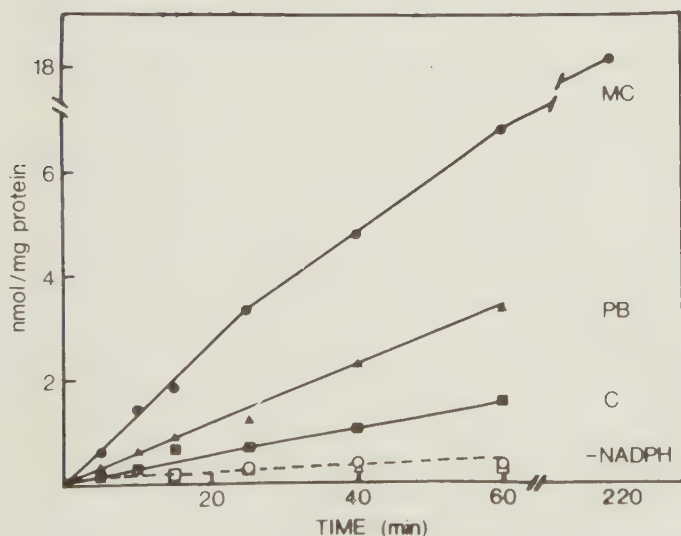


Fig. 2. Time-course of benzo[a]pyrene incorporation into microsomal proteins. Standard incubation conditions were used with liver microsomes from MC-treated (●), PB-treated (▲), and control (■) rats.

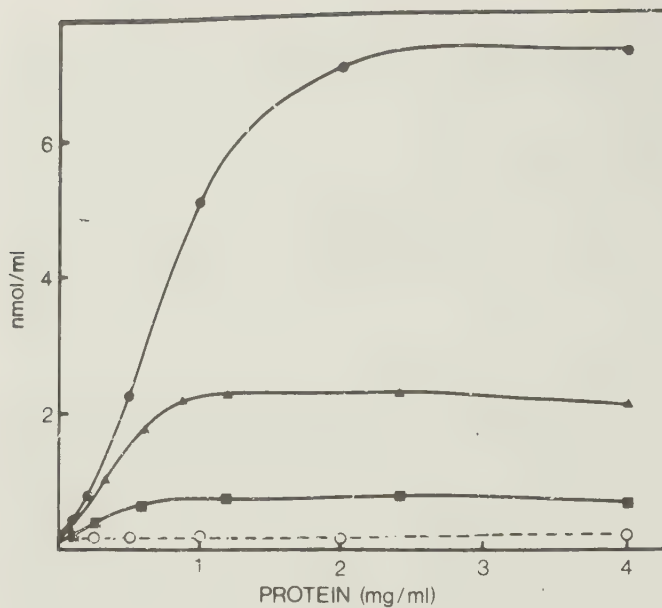


Fig. 3. The effect of protein concentration on benzo[*a*]pyrene incorporation. Standard incubation conditions with various concentrations of microsomal proteins from MC-treated (—), PB-treated (—), and control (—) rats were used. Closed symbols are incubations with and open symbols without NADPH.

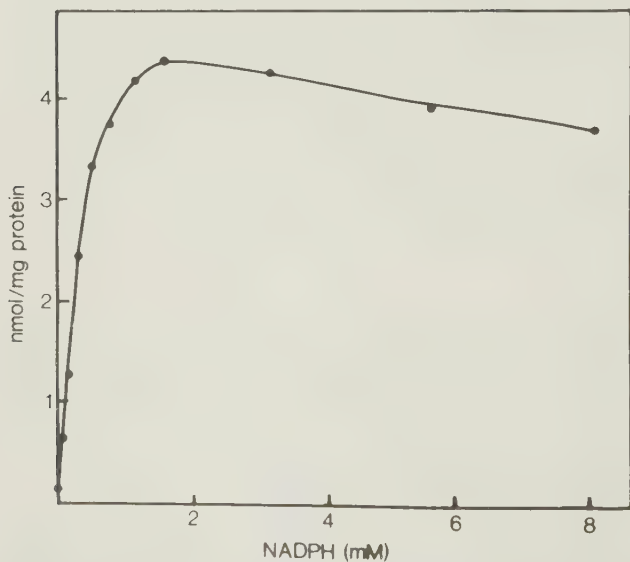


Fig. 4. The effect of NADPH on the incorporation of benzo[*a*]pyrene. Standard incubation conditions with liver microsomes from MC-treated rats and increasing concentrations of NADPH were used.

microsomal protein from MC-treated rats between 0.2–1 mg/ml incubation mixture. The incorporation was comparatively low below this interval, and was independent of added protein above 2 mg/ml. Similar curves were obtained with microsomes from control or PB-treated rats, although the incorporation was much lower in these cases.

The dependency on the concentration of NADPH for the incorporation of benzo[a]pyrene into microsomal proteins is shown in Fig. 4. The incorporation increased with increasing concentrations of NADPH reaching a maximum at 1.6 mM. A slight inhibition of the reaction was observed at higher concentrations. As an alternative we tested the effect of a NADPH-generating system [18]. Then the incorporation was approx. 30% of that obtained with an optimal concentration of NADPH. Obviously the system did not generate a sufficient steady-state concentration of NADPH, although the concentration of NADP was 0.85 mM and most of this is supposed to be kept in the reduced state during the incubation.

The influence of the benzo[a]pyrene concentration on the extent of incorporation was also examined (Fig. 5). A maximum binding was obtained at a concentration of 25 μ M. This concentration is similar to that yielding a maximum incorporation of benzo[a]pyrene into nuclear proteins [17], but is only half of that yielding a maximum benzo[a]pyrene mono-oxygenase activity [26].

Incorporation into microsomal RNA

The possibility that benzo[a]pyrene or its metabolites was incorporated into microsomal RNA rather than into proteins was also considered. The specific binding of benzo[a]pyrene was 31 pmol/mg RNA in a standard

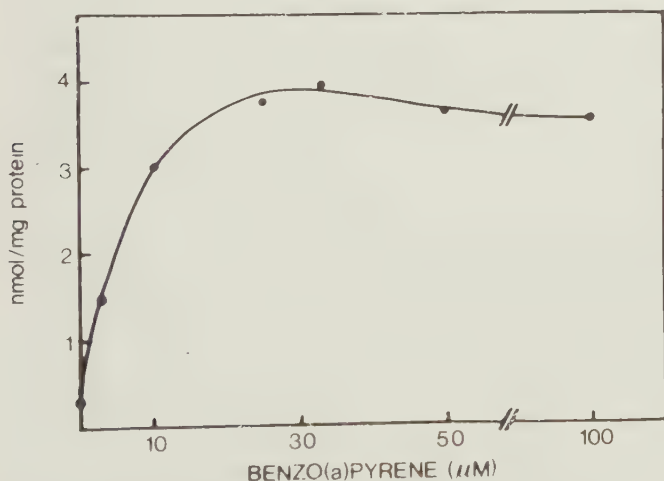


Fig. 5. The effect of benzo[a]pyrene concentration on its incorporation into microsomal proteins. Standard incubation conditions with liver microsomes from MC-treated rats and increasing concentrations of benzo[a]pyrene were used.

incubation as compared to 4000 pmol/mg microsomal proteins. Since the ratio of protein to RNA in these microsomes was 7 : 1 (w/w), only 0.1% of the benzo[a]pyrene that bound to macromolecules was incorporated into RNA under our incubation conditions.

DISCUSSION

The presented method allows a simple, rapid, sensitive and accurate quantitative analysis of the covalent binding of xenobiotics to proteins. The binding of benzo[a]pyrene and its metabolites to microsomal proteins has been examined here, but the method can be extended to measure the covalent binding of other radiolabelled xenobiotics to proteins from various subcellular fractions provided that unbound radiolabelled substances can be removed from the filter paper discs without dissolving the protein fraction. For instance, we have used the method to study the incorporation of metabolically activated benzo[a]pyrene to cytosolic proteins (Schelin et al., in prep.). The method can be used both for ^3H -labelled and ^{14}C -labelled compounds, although the counting recovery of ^3H is much lower than of ^{14}C (53% and 96%, respectively, in our scintillation counter), as is the counting efficiency.

The analysis of protein-bound chloroform after precipitation with trichloroacetic acid on filter papers or glassfibre filters followed by trichloroacetic acid and alcohol washes has been described [20,27]. This precipitation and washing procedure was not efficient, however, for a polycyclic aromatic hydrocarbon as benzo[a]pyrene.

An advantage with our method is its sensitivity. The incorporation of benzo[a]pyrene to microsomal proteins from MC-treated rats could be measured accurately using 10 μg of microsomal protein (0.1 mg/ml in the incubation mixture). In comparison, several mg of microsomal proteins have been used in other studies [11,15,16], presumably due to the requirement of bulk proteins for a good recovery during the extraction procedure. This is not a problem when using the present method since the proteins are kept precipitated on the filters during the entire washing. Since the incorporation of benzo[a]pyrene (and presumably also of other xenobiotics) is not proportional to the concentration of microsomal proteins above 1–2 mg/ml (which is a much lower protein concentration than what has been used in other binding studies) this new method should allow a more accurate analysis of the specific incorporation of xenobiotics into proteins.

The washing procedure of the filters may be simplified by omitting one or more of the steps. This tends, however, to give a higher background and less reproducible results. If the incorporation is considerably higher than the background this may not be a serious drawback. We have found, for instance, that one methanol and the *n*-hexane washing step can be omitted if maximum accuracy is not required. When the incorporation of other xenobiotics than benzo[a]pyrene is examined, changes in the washing procedure may be necessary. For instance, polar substances may need more extensive washing in polar solvents and non-polar ones in non-polar solvents.

As the precipitation and washing procedure is outlined here a quantitative recovery of DNA and RNA is also obtained. It should be possible to extract the nucleic acids from the filters by altering the procedure. This should be of interest when the binding of xenobiotics to nuclear components is examined.

The covalent nature of the binding between radiolabelled benzo[a]pyrene and proteins is suggested by several pieces of evidence. The bound radiolabel cannot be removed from the filter paper discs by organic solvents or other protein precipitants we have tested, such as trichloroacetic acid. This, together with the metabolic activation required for binding and the efficient extraction of both benzo[a]pyrene and isolated benzo[a]pyrene metabolites from the filter papers, precludes even a high-affinity non-covalent binding between benzo[a]pyrene and the precipitated proteins (or the filters). We also know from our earlier experiments [9,10] that both benzo[a]pyrene and metabolites of benzo[a]pyrene will migrate with protein fractions on electrophoresis after denaturation with SDS.

The binding of benzo[a]pyrene to microsomal proteins under our incubation conditions is rather extensive. The maximum binding we have observed, close to 20 nmol bound per milligram protein, corresponds to a unity molar ratio between incorporated benzo[a]pyrene and microsomal proteins, assuming an average molecular weight of 50 000 for the proteins. Then approx. 70% of the added benzo[a]pyrene had bound to proteins, either after one or more cycles of metabolic activation (cf. Ref. 10). Studies of the binding of other xenobiotics to proteins after incubations with microsomes show similar high levels of incorporation using other methods of analysis [11,15,19].

In summary, the presented method allows an accurate analysis of the incorporation of small amounts of metabolically activated xenobiotics to proteins of various subcellular fractions. Because of its sensitivity the method should be useful in studies on the incorporation of xenobiotics to specific target proteins, whether isolated or in microsomes or other subcellular fractions.

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II

COVALENT BINDING OF BENZO[*a*]PYRENE TO CYTOCHROME P-450_{βNF-B2} AND OTHER PROTEINS IN RECONSTITUTED MIXED-FUNCTION OXIDASE SYSTEMS

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SUMMARY

A reconstituted mixed-function oxidase system, containing the major β -naphthoflavone-induced isozyme of rat liver cytochrome *P*-450 bound benzo[*a*]pyrene covalently in the presence of NADPH. NADPH-cytochrome *P*-450 reductase was required for binding and a maximum rate of adduct formation was obtained at 8 units of reductase per nmol cytochrome *P*-450. Phosphatidylcholine inhibited this reaction. Benzo[*a*]pyrene was bound to the cytochrome, but not to the reductase, as shown by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Approximately 6 molecules of benzo[*a*]pyrene bound to each molecule cytochrome *P*-450 during prolonged incubations. No binding occurred when the β -naphthoflavone-induced isozyme of cytochrome *P*-450 was replaced by the major isozyme induced by phenobarbital, but both cytochromes incorporated benzo[*a*]pyrene to approximately the same extent when they were incubated together in the presence of the reductase and NADPH. Metabolically activated benzo[*a*]pyrene also bound covalently to purified epoxide hydrolase, when this enzyme was added to the reconstituted mixed-function oxidase system.

Key words: Benzo[*a*]pyrene — Covalent binding — Cytochrome *P*-450 — Mixed function oxidase

INTRODUCTION

Many carcinogenic and mutagenic polycyclic aromatic hydrocarbons, including benzo[*a*]pyrene, are converted to electrophilic metabolites by the

Abbreviations: cytochrome *P*-450_{βNF-B2}, the major form induced by β -naphthoflavone; cytochrome *P*-450_{PB-B2}, the major form induced by phenobarbital; HPLC, high-pressure liquid chromatography; SDS, sodium dodecylsulfate.

microsomal mixed-function oxidase system. Most of these metabolites are converted further to more polar species than can be excreted readily from the cell [1,2]. The reactive metabolites may also bind covalently to cellular macromolecules [1-4]. This binding has been implicated in the toxic, mutagenic and carcinogenic properties of these compounds [5-9]. The binding of metabolites to DNA has been studied extensively due to its possible connection with carcinogenicity [2,4,10]. The binding to proteins, on the other hand, which in the case of polycyclic aromatic hydrocarbons by far exceeds the binding to DNA or RNA in various cells [7,11] has not been examined in detail. As a consequence, the physiological significance of the binding to proteins is less well known, although it has been implicated in cell toxicity [3,5,7,8].

It is now established that after metabolic activation polycyclic aromatic hydrocarbons and other toxic substances bind specifically rather than randomly to proteins in various subcellular fractions. Thus, histones have been identified as target proteins in the nucleus [3,7,12] and glutathione transferase B (ligandin) in the cytosol [3,13-16]. As for microsomes, i.e. the site of metabolite activation of these compounds, little is known about the identity of the target proteins. Enzymes in the mixed-function oxidase system have been suggested to bind xenobiotics covalently [17-22]. Chloramphenicol and parathion have been shown to form adducts with cytochrome P-450 after metabolic activation by a reconstituted mixed-function oxidase system [23,24] and this binding causes an inactivation of the cytochrome.

In order to identify target proteins for benzo[*a*]pyrene, we have incubated this hydrocarbon with purified microsomal enzymes which are part of the mixed-function oxidase system. We present evidence that cytochrome P-450 as well as epoxide hydrolase will bind metabolically activated benzo[*a*]pyrene.

MATERIALS AND METHODS

Chemicals

[¹⁴C]Benzo[*a*]pyrene (21.7 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. 2',5'-ADP-Sepharose 4B was from Pharmacia Fine Chemicals, L- α -phosphatidylcholine type V-E from Sigma, Rénex 690 from Kemi-Intressen, Sundbyberg, Sweden and Omnifluor and Enlightning from New England Nuclear. All other chemicals used were of analytical grade and purchased from common commercial sources.

Preparation of microsomes

Male Sprague Dawley rats (Anticimex, Stockholm) weighing 150-200 g were given β -naphthoflavone (40 mg/kg) in 0.5 ml corn oil intraperitoneally for 3 days before sacrifice, or phenobarbital (0.1% in the drinking water) for 5 days before sacrifice. Microsomes were prepared as described earlier [23].

Purification of NADPH-cytochrome P-450 reductase

The enzyme was isolated from rats pretreated with phenobarbital using the following modifications of the method of Yasukochi and Masters [25]. One volume of microsomal suspension was solubilized in 3 vol. of 25 mM Tris-HCl (pH 7.7), 10 mM EDTA, 0.1 mM dithioerythritol, 0.5% sodium cholate, and 1.6% Renex 690. After centrifugation at $105\,000 \times g$ for 90 min, the solubilized material was applied directly to a 2',5'-ADP-Sepharose 4B column previously equilibrated with 0.1 M potassium phosphate buffer (pH 7.7), 0.4 mM EDTA, 0.1 mM dithioerythritol, 0.1% Renex 690 and 24% glycerol. After washing the column the reductase was eluted with 1 mM 2'-AMP in the same buffer mixture. Fractions containing reductase were combined and treated with charcoal, SiO₂ (diatomaceous earth) and Amberlite XAD-2 to remove 2'-AMP and detergents. The final preparation had a specific activity of 25 units of NADPH-cytochrome P-450 reductase per mg protein. The enzyme was homogeneous as judged by SDS-polyacrylamide gel electrophoresis. One unit is defined as the amount of enzyme catalyzing the reduction of 1 μ mol cytochrome *c* per min at 22°C.

Purification of cytochrome P-450 and epoxide hydrolase

Epoxide hydrolase and the major forms of cytochrome P-450 from liver microsomes of phenobarbital-(cytochrome P-450_{PB-B2}) and β -naphthoflavone-(cytochrome P-450 _{β NF-B2}) treated rats were purified as described by Guengerich and Martin [26] with the following modifications: After sample application the octylamino-Sepharose column was washed with buffer containing 0.5% rather than 0.42% sodium cholate. The cytochrome P-450 was then eluted with buffer containing 0.08% rather than 0.06% Lubrol PX and 0.40% rather than 0.33% sodium cholate. The cytochrome P-450 fraction from octylamino-Sepharose was chromatographed on a 2 $\times$ 90 cm column of DEAE-Sephacel. A 1.5- ℓ linear gradient of 25–100 mM NaCl in buffer was used to elute the various P-450 forms. The altered conditions were necessary to separate the major P-450 fractions (B2) from a later fraction of slightly higher molecular weight (B3) present in the microsomes from both the phenobarbital- and β -naphthoflavone-treated rats.

The B2 fractions and the epoxide hydrolase fraction II used in this study were >95% pure as judged by SDS polyacrylamide gel electrophoresis (cf. Fig. 5). The specific heme content of these cytochrome P-450 preparations was 15 nmol/mg protein, and the specific 7-ethoxycoumarin deethylase activity was 25 and 85 nmol 7-hydroxycoumarin formed per min and nmol heme for the PB-B2 and β NF-B2 forms, respectively [23]. The specific activity of the epoxide hydrolase used was 215 nmol styrene glycol hydrolysed per min and mg protein [27].

Quantitative determination of covalently bound benzo[a]pyrene

The covalent binding of benzo[a]pyrene to proteins was analyzed as described in detail by Wallin et al. [28]. The incubation mixtures contained in a

final volume of 200 μ l, 50 mM Tris-HCl (pH 7.4), 5 mM MgCl_2 , 2 mM NADPH and 25 μ M benzo[a]pyrene. Cytochrome P -450_{PB-B2} or P -450 _{β NF-B2}, NADPH-cytochrome P -450 reductase, epoxide hydrolase and phosphatidylcholine were added as indicated in each case. The incubation time was 30 min, unless otherwise indicated. After incubation the proteins were precipitated on filter paper discs and unbound benzo[a]pyrene was extracted before analysis by scintillation counting. Some incubations were performed in the presence of 0.1 mM α -naphthoflavone, 0.1 mM metyrapone or 2 mM NADH.

Analysis of protein-binding patterns by electrophoresis

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [29] using 8% slab gels. The incubation mixtures had the same composition as for quantitative analyses described above. In addition, 0.333 units NADPH-cytochrome P -450 reductase, 0.025 nmol cytochrome P -450, 0.125 nmol epoxide hydrolase or 50 μ g microsomal proteins was added as indicated in each case. After incubation for 30 min an equal volume of denaturation mixture, containing 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol and 10% β -mercaptoethanol, was added and the samples were boiled for one min. After electrophoresis gels were stained overnight in 0.1% Coomassie Brilliant Blue dissolved in 50% methanol and 7% acetic acid. After destaining in 25% methanol and 7% acetic acid the gels were soaked in 5 volumes of Enlightning for 30 min. The gels were dried under vacuum with heat, and exposed to Kodak X-Omat films at -70°C for 3 weeks. Molecular weight standards used were bovine serum albumin ($M_r = 69\,000$), ovalbumin ($M_r = 45\,000$) and carbonic anhydrase ($M_r = 30\,000$).

Analytical procedures

Protein was determined by the Lowry method [30] using bovine serum albumin as standard. Cytochrome P -450 was determined according to Omura and Sato [31], and NADPH-cytochrome P -450 reductase (using cytochrome c as substrate) according to Hrycay and O'Brien [32]. The analyses were performed in duplicate.

RESULTS

Binding of benzo(a)pyrene to mixed-function oxidase components

The possibility that benzo[a]pyrene would bind covalently to the components of a reconstituted mixed-function oxidase system consisting of cytochrome P -450, NADPH-cytochrome P -450 reductase and phosphatidylcholine was examined first (Fig. 1). Binding, increasing linearly with time for approx. 30 min, occurred in the system containing cytochrome P -450 _{β NF-B2}. The binding was dependent on added NADPH, indicating a requirement for metabolic activation of the benzo[a]pyrene. In contrast, there was no binding to components in a system containing cytochrome P -450_{PB-B2}.

Effect of NADPH-cytochrome P-450 reductase

The effect of the amount of NADPH-cytochrome P-450 reductase on the covalent binding of benzo[a]pyrene in the reconstituted system is shown in Fig. 2. No binding occurred in the absence of reductase, but binding increased with increasing concentrations of the enzyme reaching an optimum at approximately 0.4 U of reductase added (8 units/nmol cytochrome P-450).

Effect of phosphatidylcholine

The metabolism of a number of substrates including benzo[a]pyrene [33,34] in reconstituted mixed-function oxidase systems is dependent on added phospholipids. The effect of phosphatidylcholine on the covalent binding of benzo[a]pyrene to components of reconstituted systems was therefore examined (Fig. 3). The highest binding was obtained when no phospholipid was added, whereas the binding was inhibited up to 50% by increasing concentrations of phosphatidylcholine.

Cytochrome P-450 concentrations

The effect of the amount of cytochrome P-450 on the rate of benzo[a]pyrene binding in the reconstituted system was examined using a fixed molar ratio of reductase to cytochrome (Fig. 4). The initial rate of binding was proportional to the amount of cytochrome P-450_{βNF-B2} added up to 0.05 nmol. When this cytochrome was replaced by cytochrome P-450_{PB-B2} the binding was negligible even at high concentrations of enzymes, thus corroborating the results of Fig. 1.

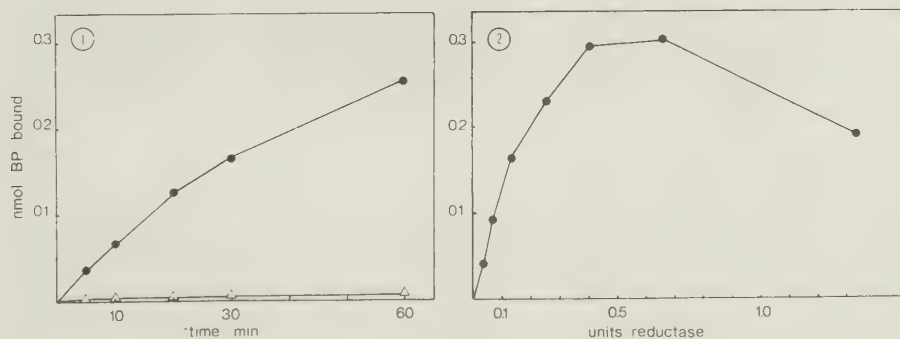


Fig. 1. Time course of the covalent binding of benzo[a]pyrene to isolated proteins in reconstituted systems. Cytochromes P-450_{PB-B2} (Δ) or P-450_{βNF-B2} ($\bullet$) (0.05 nmol) was incubated with 0.13 units NADPH-cytochrome P-450 reductase and 10 μ g phosphatidylcholine as described in Methods.

Fig. 2. Effect of NADPH-cytochrome P-450 reductase on the covalent binding of benzo[a]pyrene to protein. The incubation mixtures contained 0.05 nmol cytochrome P-450_{βNF-B2}, 10 μ g phosphatidylcholine and various amounts of reductase.

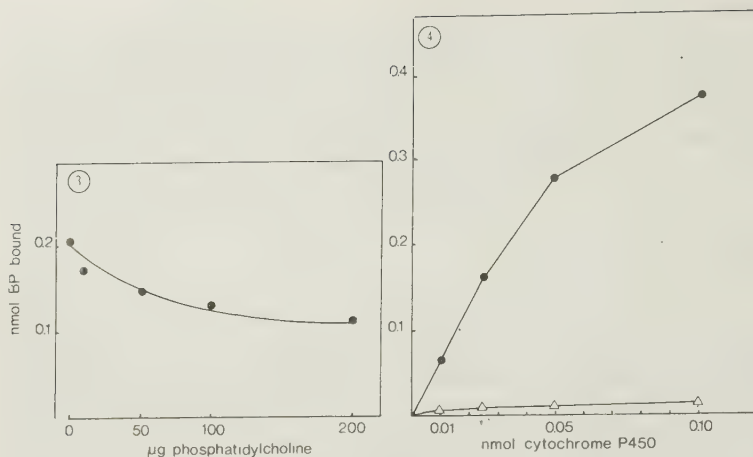


Fig. 3. Effect of phosphatidylcholine on the covalent binding of benzo[a]pyrene to proteins. The incubation mixtures contained 0.05 nmol cytochrome $P-450_{\beta NF-B2}$, 0.13 units NADPH-cytochrome $P-450$ reductase and various amounts of phosphatidylcholine.

Fig. 4. Effect of the amount of cytochrome $P-450$ on the covalent binding of benzo[a]pyrene to proteins in reconstituted systems. The incubation mixtures contained NADPH-cytochrome $P-450$ reductase in a molar excess of 8 to the cytochrome, 10 μ g phosphatidylcholine, and the amount of cytochrome $P-450_{PB-B2}$ (Δ) or $P-450_{\beta NF-B2}$ ($\bullet$) indicated.

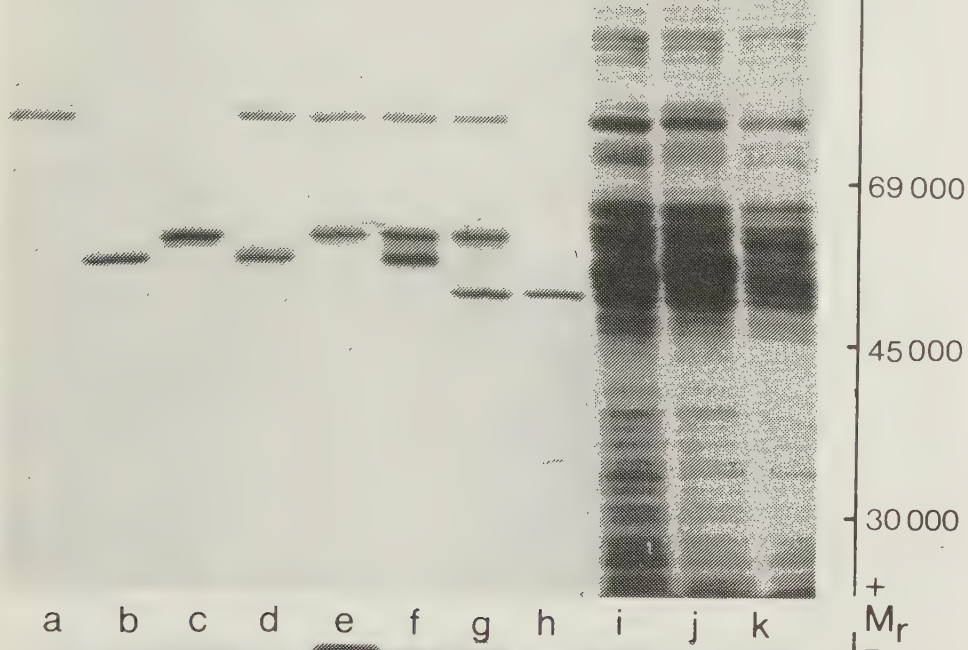
Effect of NADH and inhibitors of cytochrome $P-450$ activity

When NADPH was replaced by NADH in the incubation mixture, the binding of benzo[a]pyrene decreased by more than 90% (Table I). A similar low degree of binding was observed in the presence of α -naphthoflavone, an inhibitor of the 3-methylcholanthrene-induced form of cytochrome $P-450$ [35]. Metyrapone, an inhibitor of the phenobarbital-induced form of cytochrome $P-450$ [36], also inhibited benzo[a]pyrene binding in the reconstituted system containing cytochrome $P-450_{\beta NF-B2}$, but to a somewhat smaller extent than α -naphthoflavone did.

Protein targets for benzo(a)pyrene

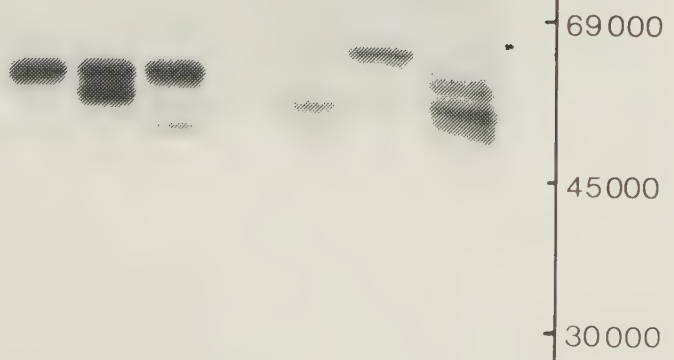
The identity of the protein targets for the metabolically activated benzo[a]pyrene was examined by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 5). When a reconstituted system was incubated with NADPH the covalent binding of radiolabelled benzo[a]pyrene occurred to cytochrome $P-450_{\beta NF-B2}$, whereas there was no binding in the position of

Fig. 5. Protein patterns and autoradiographs after SDS-polyacrylamide gel electrophoresis. (a) NADPH-cytochrome $P-450$ reductase; (b) cytochrome $P-450_{PB-B2}$; (c) cytochrome $P-450_{\beta NF-B2}$; (d) reductase and cytochrome $P-450_{PB-B2}$; (e) reductase and cytochrome $P-450_{\beta NF-B2}$; (f) reductase, cytochrome $P-450_{PB-B2}$ and $P-450_{\beta NF-B2}$; (g) reductase, cytochrome $P-450_{\beta NF-B2}$ and epoxide hydrolase; (h) epoxide hydrolase; (i) control liver microsomes; (j) microsomes from phenobarbital-treated rats; (k) microsomes from β -naphthoflavone-treated rats.



a b c d e f g h i j k

+
M_r
-



69 000
45 000
30 000
+

TABLE I

EFFECT OF COFACTORS AND INHIBITORS ON THE COVALENT BINDING OF BENZO[a]PYRENE TO PROTEINS IN A RECONSTITUTED MIXED-FUNCTION OXIDASE SYSTEM

Incubations were performed in the presence of 0.05 nmol cytochrome $P-450_{\beta\text{NF-B2}}$ 0.13 units NADPH-cytochrome $P-450$ reductase and 10 μg phosphatidylcholine as described in Methods.

Additions	Conc. (mM)	Benzo[a]pyrene bound (pmol)
Control		161
NADH (-NADPH)	2	13
α -Naphthoflavone	0.1	10
Metyrapone	0.1	47

NADPH-cytochrome $P-450$ reductase (lane e). As expected, cytochrome $P-450_{\text{PB-B2}}$ did not form adducts with benzo[a]pyrene when incubated in place of $P-450_{\beta\text{NF-B2}}$ in the reconstituted system (lane d). When the cytochromes were incubated together, however, binding occurred to both of them (lane f) and interestingly enough both cytochromes bound benzo[a]pyrene to approximately the same extent. This shows that target groups are present in cytochrome $P-450_{\text{PB-B2}}$, but that this cytochrome does not catalyze the formation of benzo[a]pyrene metabolites able to react with these groups. No radioactivity was present in the position of NADPH-cytochrome $P-450$ reductase in any of the incubations, yet this enzyme had to be present for the formation of protein adducts (lanes b and c).

The possibility that epoxide hydrolase could be a target for activated benzo[a]pyrene was also examined. Covalent binding to this enzyme was observed in the presence of cytochrome $P-450_{\beta\text{NF-B2}}$ and reductase (lane g). The extent of binding to epoxide hydrolase was, however, less than that to the cytochrome.

As a comparison with the reconstituted systems, the binding of benzo[a]pyrene to components of liver microsomes from control, phenobarbital- and 3-methylcholanthrene-pretreated rats is also shown (lanes i-k). A radiolabeled band is seen in the position of cytochrome $P-450_{\beta\text{NF-B2}}$ in the microsomes from β -naphthoflavone-pretreated animals, together with a band in the position of epoxide hydrolase and a strong intermediate band. Benzo[a]pyrene bound predominantly to one component in liver microsomes from phenobarbital-pretreated rats. This component had a higher molecular weight than cytochrome $P-450_{\text{PB-B2}}$ and did not correspond to any labelled component in microsomes from 3-methylcholanthrene-pretreated rats. These radiolabeling patterns verify our earlier observations [17].

A rather strong and diffuse zone of radiolabel was observed at the top of the fluorographs. Since there was no corresponding protein staining, this was probably due to benzo[a]pyrene or uncharged benzo[a]pyrene metabolites not removed in the electrophoretic procedure.

DISCUSSION

We have shown here that cytochrome $P-450_{\beta\text{NF-B}_2}$ in the presence of NADPH-cytochrome $P-450$ reductase and NADPH will bind benzo[*a*]-pyrene covalently, whereas the reductase is not a target for the aromatic hydrocarbon. We have also shown that epoxide hydrolase and cytochrome $P-450_{\text{PB-B}_2}$, two other enzymes involved in the metabolism of xenobiotics, may form adducts with benzo[*a*]pyrene under appropriate conditions. The requirement for reductase and NADPH together with cytochrome $P-450$ showed that metabolic activation of benzo[*a*]pyrene preceded binding. This was further suggested by the low level of adduct formation observed in the presence of the cytochrome $P-450$ inhibitors, or when NADPH was replaced by NADH. It was also evident that cytochrome $P-450_{\text{PB-B}_2}$ did not catalyze the formation of benzo[*a*]pyrene metabolites that could react with this cytochrome, since there was no binding in the absence of cytochrome $P-450_{\beta\text{NF-B}_2}$. However, it should be pointed out that the cytochrome $P-450_{\text{PB-B}_2}$ preparation used is quite active in the metabolism of benzo[*a*]pyrene, producing 7 nmol of metabolites per min and nmol as determined by HPLC [37].

A previous study by Gozukara et al. [38] has shown that benzo[*a*]pyrene-7,8-dihydrodiol will form adducts with proteins of the mixed-function oxidase system after metabolic activation. In this case rabbit liver phenobarbital-induced cytochrome $P-450$ catalyzed the reaction. Covalent binding occurred to this protein and to all other proteins included in the reaction mixtures, i.e. NADPH-cytochrome $P-450$ reductase, epoxide hydrolase and bovine serum albumin. Thus the target specificity of the dihydrodiol was less than that of activated benzo[*a*]pyrene in this study. One possible reason for this is that the dihydrodiol is metabolized to the highly reactive 9,10-oxide which will react readily with various macromolecules [1]. We have found earlier that after metabolic activation the dihydrodiol will bind more efficiently and less specifically to rat liver microsomal proteins than do several other metabolites of benzo[*a*]pyrene [39].

So far we have not determined the ultimate metabolite(s) of benzo[*a*]pyrene that become bound to the mixed-function oxidase proteins. The 7,8-dihydrodiol-9,10-epoxide is a strong candidate, since it is a major reaction product of cytochrome $P-450_{\beta\text{NF-B}_2}$ [40], but not of cytochrome $P-450_{\text{PB-B}_2}$, and since it will readily form adducts with macromolecules. The previously suggested benzo[*a*]pyrene-4,5-oxide [41] is less likely to be a major binding species here, since it is formed preferentially by cytochrome $P-450_{\text{PB-B}_2}$, and no binding was observed with this cytochrome. It is possible, however, that several benzo[*a*]pyrene metabolites bind covalently to proteins in reconstituted systems, although with different efficiencies, as suggested by the binding studies of isolated benzo(a)pyrene metabolites to microsomes [39].

Apart from benzo[*a*]pyrene, other substances have also been shown to bind covalently to isolated cytochrome $P-450$. Both parathion [24] and

chloramphenicol [23] formed adducts primarily with amino acid residues of cytochrome *P*-450_{PB-B2} after metabolic activation by a reconstituted mixed-function oxidase system. These modifications led to an inactivation of the cytochrome. Whether covalently bound benzo[*a*]pyrene would have a similar effect on the activity of cytochrome *P*-450_{βNF-B2} is not known presently, but the linear time course of covalent binding (Fig. 1) might argue against such a possibility.

The covalent binding of benzo[*a*]pyrene to cytochrome *P*-450 preferentially occurred to the protein moiety as judged by the electrophoretic behaviour of the radiolabel. The amino acid residues involved in the binding are not known. They are likely to be nucleophiles, however, since reactive metabolites are almost always electrophiles. Thus, lysine appeared to be involved in the binding of chloramphenicol [23,24], as well as in the binding of activated benzo[*a*]pyrene to histone H1 [42]. In addition, methionine is a target for metabolites of acetylaminofluorene and azo dyes [43]. Also cysteine and histidine are likely to be targets for electrophilic attack [44,45].

Earlier studies have shown that there is little metabolism of benzphetamine in reconstituted mixed-function oxidase systems in the absence of phospholipids [34]. The covalent binding of benzo[*a*]pyrene was inhibited, however, by added phosphatidylcholine. This might be due to a different effect of phospholipids on the metabolism of the more hydrophobic substrate benzo[*a*]pyrene. It seems less likely that enough phospholipids were present in the enzyme preparations to obtain a maximum metabolism of benzo[*a*]pyrene if the same amount of phospholipids were required as for the metabolism of benzphetamine [34].

The maximum rate of benzo[*a*]pyrene binding in the reconstituted system was approx. 0.1 nmol/min and nmol cytochrome *P*-450_{βNF-B2}. This is slightly lower than the rate observed with liver microsomes from 3-methylcholanthrene-treated rats [28]. The amount of benzo[*a*]pyrene bound on prolonged incubations was more than 6 molecules per molecule cytochrome *P*-450_{βNF-B2}. The effect of this very high degree of substitution on the properties of the cytochrome is presently being investigated.

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III

EVIDENCE THAT COVALENT BINDING OF METABOLICALLY ACTIVATED PHENOL
TO MICROSOMAL PROTEINS IS CAUSED BY OXIDISED PRODUCTS OF
HYDROQUINONE AND CATECHOL.

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Summary.

The metabolic activation of [^{14}C]phenol resulting in covalent binding to proteins has been studied in rat liver microsomes. The covalent binding was dependent on microsomal enzymes and NADPH and showed saturation kinetics for phenol with a K_m of 0.04 mM. The metabolites hydroquinone and catechol were formed at rates which were 10 or 0.7 times that of the binding rate of metabolically activated phenol. The effects of cytochrome P-450 inhibitors and cytochrome P-450 inducers on the metabolism and binding of phenol to microsomal proteins, suggest that cytochrome P-450 isoenzyme(s) other than P-450_{pg-B} or P-450_{βNF-B} catalyses the metabolic activation of phenol. Furthermore, reconstituted mixed-function oxidase systems containing cytochrome P-450_{pg-B} and P-450_{βNF-B} were (on basis of cytochrome P-450 content) 6 and 11 times less active in catalysing the formation of hydroquinone than microsomes. The isolated metabolites hydroquinone and catechol bound more extensively to microsomal proteins than phenol and the binding of these was not stimulated by NADPH. The binding occurring during the metabolism of phenol could be predicted by the rates of formation of hydroquinone and catechol and the rates by which the isolated metabolites were bound to proteins.

Introduction.

Exposure to benzene may cause leukemia and aplastic anemia in man (1). Although the mechanism behind benzene toxicity is unknown, reactive metabolites have been implied in causing toxicity (1). The liver is the major site of benzene metabolism (2), and it has been suggested (3) that metabolites are transported from the liver to bone marrow, which is the prime target organ in benzene toxicity (1)

In liver benzene is metabolized to its epoxide which is rearranged to phenol. However, steady state concentration and the reactivity of benzenoxide are too low to explain the covalent binding of benzene which occurs to proteins in liver microsomes, while phenol, the major metabolite of benzene, is bound to a considerable extent (4). A recent study (5) showed that phenol had to be metabolized before binding covalently to liver microsomal proteins, and that the metabolites hydroquinone and catechol were formed. In this report we have further investigated the mechanism of metabolic activation of phenol.

Materials and methods.

Chemicals.

[^{14}C] Phenol (104 mCi/mmol) was obtained from Amersham International. [^{14}C] Labelled hydroquinone and catechol were synthesised as described below. All chemicals were of analytical grade.

Microsomes.

Liver microsomes were prepared from adult male Sprague-Dawley rats (6), either untreated or treated with phenobarbital or 3-methylcholanthrene (7,8). The microsomal content of cytochrome P-450 (9) was 0.69, 2.71 and 1.24 nmol/mg, respectively.

Covalent binding to proteins.

The standard incubation mixture contained 0.1 mg of microsomal protein from phenobarbital treated rats, 20 nmol ^{14}C phenol, 50 μmol NADPH (omitted in controls), 0.5 μmol MgCl_2 , and 5 μl Tris-HCl, pH 7.4, in 100 μl . Cofactors and inhibitors were added in buffer or (α -naphthoflavone) in 5 μl dimethylsulphoxide. Incubations were for 10 min at 37 °C. Covalent binding was determined after precipitation on filter papers as described (10). When glutathione was included in the incubation mixture, filter papers were washed additionally three times in 5 %

trichloroacetic acid to remove glutathione conjugates. The analyses were performed in triplicate.

Phenol metabolism.

The metabolites of [^{14}C]phenol, obtained in incubation mixtures (see above) scaled up to 200 μl and incubated for times indicated in each case, were analysed by high performance liquid chromatography (11). The elution of metabolites was monitored by absorbance at 280 nm and liquid scintillation counting.

Preparation of [^{14}C]hydroquinone and [^{14}C]catechol.

[^{14}C]hydroquinone was obtained from standard incubations scaled up to 1 ml and incubated for 1 h. [^{14}C]Catechol was obtained through oxidation of phenol catalysed by mushroom tyrosinase. Then, 0.4 mg of mushroom tyrosinase (Sigma Chemical Co.), 0.8 μmol [^{14}C]phenol, 1 μmol ascorbic acid, and 0.1 mmol potassium phosphate buffer, pH 7.0, were incubated in a total volume of 1 ml for 30 min at 22 °C. The reaction was stopped by the addition of 200 μl 50 mM HCl-KCl buffer, pH 2.0. The radiolabelled hydroquinone and catechol were purified by high performance liquid chromatography (11). Fractions containing these metabolites were concentrated under a stream of N_2 . The concentrations of the metabolites were determined radiometrically. The metabolites were stored under nitrogen and were used within one h after their isolation. Within this time their UV spectra were identical to those described in

the literature (12,13), and the metabolites performed homogeneously on high performance liquid chromatography (11).

Preparation of enzymes.

Cytochrome P-450_{PB-B} (18 nmol/mg) and P-450_{B~~1~~NF-B} (14 nmol/mg) were prepared as described by Guengerich (14), with minor modifications (7). NADPH-cytochrome P-450 reductase was prepared by affinity chromatography (7). On basis of spectral and electrophoretic properties, inducibility and catalytic activity the cytochrome P-450_{B~~1~~NF-B} resembles the major cytochrome P-450 induced by 3-methylcholanthrene (15).

Reconstitution of mixed function oxidase systems.

The incubation mixtures contained 10 μ mol Tris-HCl, pH 7.4, 1 μ mol MgCl₂, 0.1 μ mol NADPH (omitted in controls), 0.13 U NADPH-cytochrome P-450 reductase, 0.05 nmol cytochrome P-450, 10 μ g phosphatidylcholine and 40 nmol phenol in a volume of 200 μ l. Metabolites formed during 10 min of incubation at 37 °C were analysed as described above.

Analyses.

Protein was determined by the Lowry method (16) with bovine serum albumin as standard. Benzphetamine demethylase and 7-ethoxyresorufine deethylase activities were determined as described (14).

Results.

The ability of rat liver microsomal enzymes to catalyse the formation of hydroquinone and catechol from phenol and the covalent binding of metabolites of phenol to microsomal proteins is shown in Fig. 1. Both the rates of metabolite formation and binding to proteins was linear for 10 min and then decreased. The activities were proportional to each other over the time interval examined. The initial rate of hydroquinone formation was high compared with that of catechol formation and covalent binding of phenol metabolites to microsomal proteins (4, 0.3 and 0.4 nmol/min and mg protein, respectively). After 30 min of incubation approximately 30 % of the added phenol had been converted to hydroquinone or catechol, or had become bound to microsomal proteins. All these three activities were entirely dependent on the addition of NADPH. 1,2,4-Benzenetriol was not detected in any of the experiments presented here.

The covalent binding of phenol metabolites to proteins in liver microsomes from phenobarbital-treated rats was studied in detail. The binding was proportional to the amount of microsomal protein up to a concentration of 1.5 mg/ml (Fig. 2A). It showed saturation kinetics with increasing concentrations of phenol (Fig. 2B), and the K_m for phenol, calculated from a double reciprocal plot, was 0.04 mM. A maximum rate of binding was observed at 0.5 mM NADPH. This concentration was approximately one third of that required for maximum binding of benzo(a)pyrene to

microsomal proteins (10). NADH was less efficient as electron donor yielding 10-20 % of the covalent binding obtained with NADPH (Table 2).

The effect of various inhibitors on the covalent binding of phenol metabolites to microsomal proteins was examined (Table 2). The unselective cytochrome P-450 inhibitor SKF 525A (17) at 0.5 mM concentration inhibited the binding to proteins from control and phenobarbital -treated rats by more than 80 % and to microsomal proteins from 3-methylcholanthrene -treated ones by more than 80 %. Metyrapone, a selective inhibitor of cytochrome P-450_{PB-B}, the major form induced by phenobarbital (17), inhibited binding in all three kinds of microsomal preparations. Interestingly, the binding to 3-methylcholanthrene-induced microsomes was inhibited more efficiently than to the other microsomes (reduction by 80 and 40 %, respectively, with 0.1 mM metyrapone). On the other hand α -naphthoflavone, a selective inhibitor of cytochrome P-450_{SNF-B}, the major form of cytochrome P-450 induced by 3-methylcholanthrene (17), inhibited the binding of phenol metabolites to both phenobarbital- and 3-methylcholanthrene-induced microsomes to approximately the same extent, whereas the binding to control microsomes was unaffected. No binding to proteins in any of the microsomal preparations was seen in the presence of 10 mM reduced glutathione, presumably due to scavenging of semiquinones and benzoquinones formed (18). Ascorbic acid also inhibited the binding of metabolically activated phenol efficiently. Thus, 1 mM ascorbic acid decreased

the binding by approximately 95 % in all three kinds of microsomal preparations. In contrast, the formation of catechol and hydroquinone was inhibited only by 5-10 % (not shown). This is in agreement with the fact that ascorbic acid inhibits the oxidation of benzenediols (19).

The formation of metabolites from phenol was examined in reconstituted cytochrome P-450 mixed-function oxidase systems (Table 3). Hydroquinone was formed only in complete systems consisting of cytochrome P-450, NADPH-cytochrome P-450 reductase, NADPH and phenol. One nmol of cytochrome P-450_{PB-B} catalysed the formation of 2.4 nmol hydroquinone in 10 min, whereas half that amount was formed by cytochrome P-450_{B₁NF-B}. The amount of catechol formed in these experiments was below the detection limit. The conversion rates of phenol to products were 6 (P-450_{PB-B}) and 11 (P-450_{B₁NF-B}) times lower in reconstituted systems than in microsomes on basis of cytochrome P-450 content (cf. Table 2). Therefore the catalytic properties of the reconstituted systems were checked with respect to 7-ethoxyresorufin deethylase and benzphetamine demethylase activities (Table 3). These activities were similar to what has been reported earlier (14) and were selective for respective cytochrome P-450 isoenzyme. This shows that the low conversion rates of phenol in the reconstituted systems were not due to deficient enzymes.

The ability of isolated hydroquinone and catechol to bind to microsomal proteins was investigated. Both these substances bound

efficiently, and after 30 min of incubation approximately 50 % of the added hydroquinone and 25 % of the catechol had become irreversibly bound to proteins when the initial concentrations were 6 and 6.5 μ M, respectively (Fig. 3). The initial rate of hydroquinone binding under these conditions was 1.6 nmol per mg protein during 5 min compared with 1.0 for catechol. The binding increased with increasing concentrations of these metabolites (Fig.4), and the binding of hydroquinone remained higher than that of catechol over the concentration interval examined. The binding occurred independently of electron donors; in fact, it was inhibited up to 50 % by 0.5 mM NADPH (Fig. 4). One mM ascorbic acid or 10 mM glutathione completely inhibited the binding of both hydroquinone and catechol to microsomal proteins.

Discussion.

This report confirms that enzymes in rat liver microsomes metabolise phenol to hydroquinone, catechol and products binding irreversibly to microsomal proteins. This metabolism is most likely catalysed by the mixed-function oxidase system, since it is inhibited by cytochrome P-450 inhibitors and is dependent on NADPH.

The rate of hydroquinone formation was about half that found by Sawahata and Neal (5), whereas catechol formation and covalent binding were similar. The ratios between these three activities were constant during incubation up to 30 min. They were also

constant regardless of whether the microsomes were isolated from control animals or from rats that had been pretreated with phenobarbital or 3-methylcholanthrene. This may be an indication that the orto and para hydroxylations of phenol are catalysed by the same cytochrome P-450 isoenzyme(s).

The specific rate of phenol conversion by isolated microsomes was not affected by pretreatment of animals with phenobarbital, but it was diminished to half by pretreatment with 3-methylcholanthrene. A recalculation of the data in Table 1 taking the microsomal cytochrome P-450 concentrations into consideration, showed that binding during the metabolism of phenol was 3 to 4 times higher per unit cytochrome P-450 in control microsomes than in microsomes isolated from rats treated with the cytochrome P-450 congeners. Together with the inhibition profiles obtained with cytochrome P-450 inhibitors (Table 2), this suggests that the metabolism of phenol may be catalysed at different rates by different isoenzymes of cytochrome P-450, and that the isoenzymes preferentially induced by phenobarbital and 3-methylcholanthrene do not catalyse this reaction readily. Further evidence for this suggestion came from the reconstitution experiments. Thus, the rate of conversion of phenol to hydroquinone per unit of cytochrome P-450 was 6 and 11 times lower in mixed-function oxidase systems reconstituted with cytochrome P-450_{PB-B} and P-450 _{β NF-B} (the major isoenzymes induced by phenobarbital respectively β -naphthoflavone or 3-methylcholanthrene (15)) than in intact microsomes. The low

reaction rates were not due to low activities of the reconstituted systems per se, since they were quite active in the metabolism of benzphetamine or 7-ethoxyresorufin (cf. Table 3). However, further reconstitution experiments with isolated cytochrome P-450 isoenzymes are required to resolve the role of the different isoenzymes in the metabolism of phenol.

The incubations with isolated hydroquinone and catechol showed that these metabolites will bind covalently to microsomal proteins in NADPH-independent reactions. A theoretical value for the extent of binding of these metabolites during the metabolism of phenol catalysed by microsomal proteins could be calculated, since both the initial rates of hydroquinone and catechol formation from phenol and the binding of the isolated metabolites were linear with time. Thus, the average hydroquinone and catechol concentrations were 20 and 1.5 μM , respectively, during 10 min of incubation of phenol with microsomes (Fig. 1). The binding curves of Fig. 4 indicate that a total of 3.3 nmol of these metabolites would bind in a standard incubation during 10 min (taking the inhibition of NADPH into consideration). This agrees well with the 4 nmol bound observed experimentally when phenol was incubated with microsomes.

The molecular species reacting with proteins are probably oxidative products of hydroquinone and catechol. Semiquinones have been proposed to be the ultimate binding species of metabolically activated phenol (12). Since these molecules have

unpaired electrons, we have tried to detect them in our reaction mixtures by electron paramagnetic spectroscopy. However, semiquinones, phenoxyl radicals are not present above the detection limit (about 1 nM) in reaction mixtures containing benzene, phenol, hydroquinone, catechol or benzoquinone in concentrations up to 1 mM. Therefore, if intermediates with unpaired electrons are formed during the microsomal metabolism of phenol, they have to bind rapidly to proteins.

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Legends to figures.

Fig. 1. Time course of hydroquinone (squares), and catechol (triangles) formation and of covalent binding (circles) of metabolically activated phenol to microsomal proteins. Incubation mixtures were scaled up to 300 μ l. Aliquots of 200 μ l were used for determining the formation of hydroquinone and catechol and aliquots of 50 μ l for determining the covalent binding to proteins at indicated times.

Fig. 2. The effect of protein (A) and phenol concentrations (B) on the covalent binding of metabolically activated phenol to microsomal proteins. Incubations were as described in Materials and methods.

Fig. 3. Time courses of the covalent binding of hydroquinone and catechol to microsomal proteins. Closed circles represent incubations with and open circles without NADPH. Incubations were as described in Materials and methods except that phenol was replaced by 6 μ M hydroquinone or 6.5 μ M catechol. A, Hydroquinone; B, catechol.

Fig. 4. The effect of hydroquinone and catechol concentrations on their binding to microsomal proteins. Closed circles are incubations with and open circles without NADPH. A, Hydroquinone; B, catechol.

Table 1. Covalent binding of phenol to microsomal proteins and phenol metabolite formation catalysed by liver microsomal enzymes from untreated (C) and from phenobarbital- (Pb) or 3-methylcholanthrene- (Mc) treated rats. Standard incubations scaled up to 300 μ l were performed for 10 min as described in the methods section. Aliquots of 200 μ l were analysed by high performance liquid chromatography and 50 μ l aliquots for protein binding. Analyses were performed in triplicate, and the standard deviations are given.

Product	Specific activity (nmol/mg protein per 10 min)		
	C	Pb	Mc
Protein binding	3.9 $\pm$ 0.3	4.0 $\pm$ 0.05	2.3 $\pm$ 0.2
Hydroquinone	37 $\pm$ 0.4	38 $\pm$ 1.1	16 $\pm$ 2.0
Catechol	2.3 $\pm$ 0.7	2.7 $\pm$ 0.1	1.3 $\pm$ 0.5

Table 2. Covalent binding of phenol metabolites to microsomal proteins in the presence of various cofactors and inhibitors. Standard incubations with microsomes from phenobarbital- (Pb) or 3-methylcholanthrene- treated (Mc) and untreated (C) animals were performed in triplicates. Mean values $\pm$ S.D. are given.

Agent	Covalent binding (nmol/mg)		
	C	Pb	Mc
none	3.86 $\pm$ 0.13	3.97 $\pm$ 0.20	2.36 $\pm$ 0.08
0.5 mM NADH -NADPH	0.35 $\pm$ 0.04	0.79 $\pm$ 0.04	0.43 $\pm$ 0.09
0.1 mM α -naphthoflavone	4.52 $\pm$ 0.19	2.73 $\pm$ 0.29	1.33 $\pm$ 0.07
0.1 mM metyrapone	2.46 $\pm$ 0.07	2.33 $\pm$ 0.14	0.49 $\pm$ 0.03
0.5 mM SKF 525 A	0.65 $\pm$ 0.12	0.76 $\pm$ 0.11	0.16 $\pm$ 0.04
10 mM glutathione	0.03 $\pm$ 0.11	0.04 $\pm$ 0.12	0.02 $\pm$ 0.07
1 mM ascorbic acid	0.19 $\pm$ 0.04	0.29 $\pm$ 0.03	0.12 $\pm$ 0.06

Table 3. Activities of reconstituted mixed-function oxidase systems. 7-Ethoxyresurofin and benzphetamine activities were measured as described by Guengerich et al. (14). The conversion of phenol to hydroquinone and catechol was measured as described in Materials and methods. Values given are averages of two experiments.

Substrate	specific activity (nmol of product/min per nmol of P-450)	
	P-450 _{PB-B}	P-450 _{βNF-B}
7-Ethoxyresurofin	<0.05	5.6 $\pm$ 0.3
Benzphetamine	61.5 $\pm$ 3.6	13.1 $\pm$ 3.6
Phenol		
to hydroquinone	0.24 $\pm$ 0.04	0.12 $\pm$ 0.03
to catechol	<0.02	<0.02

Fig. 1

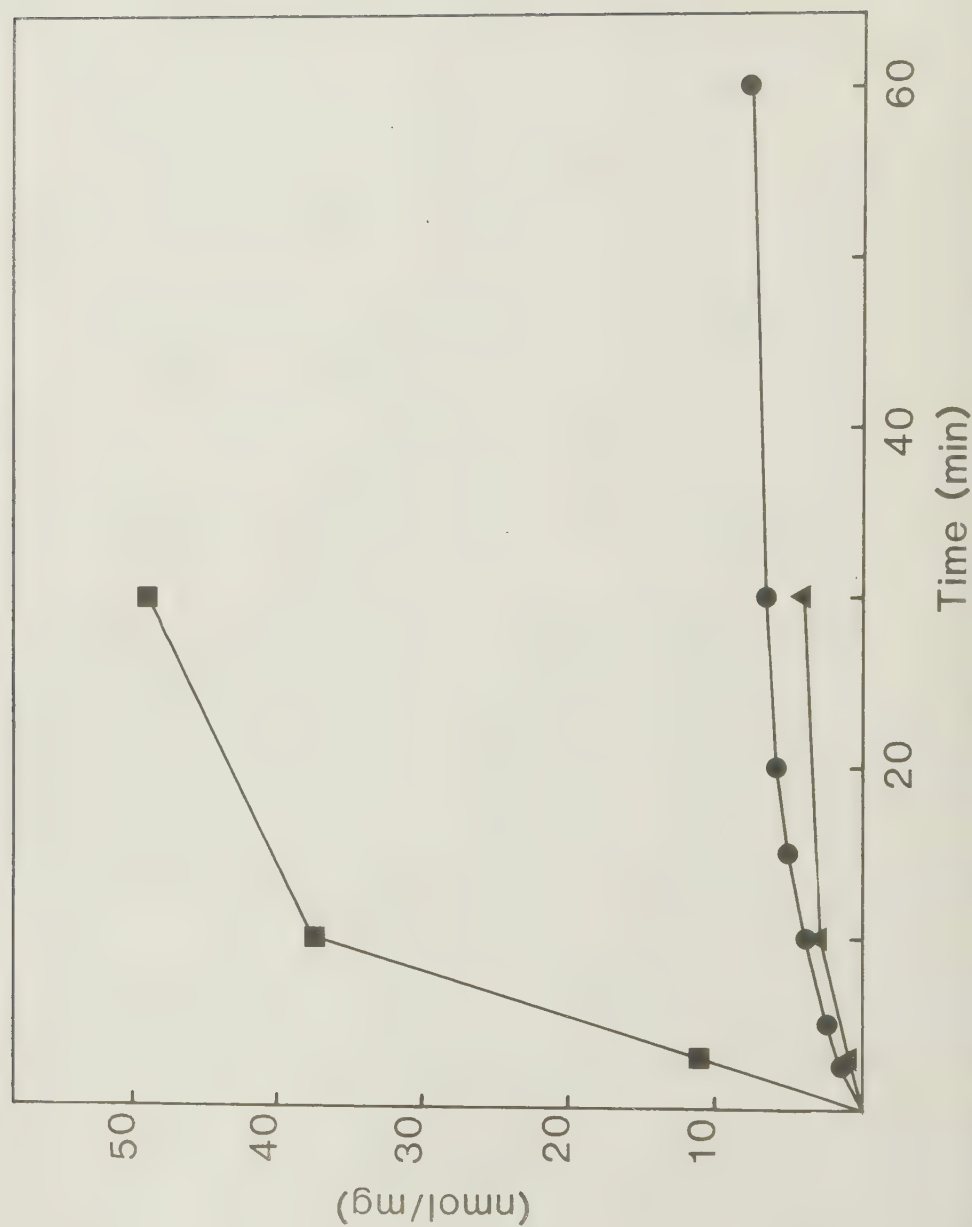


Fig. 2A

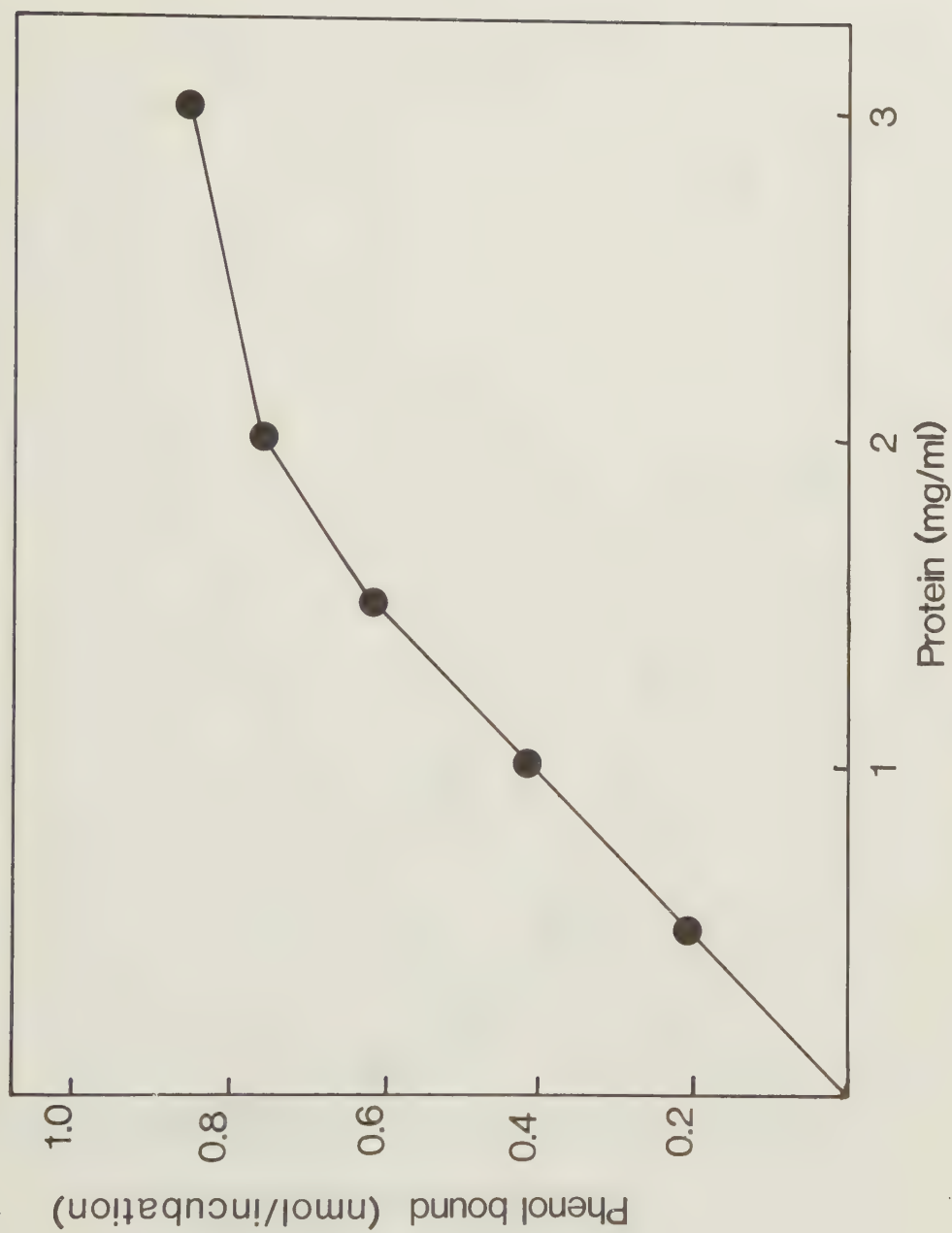


Fig. 2B

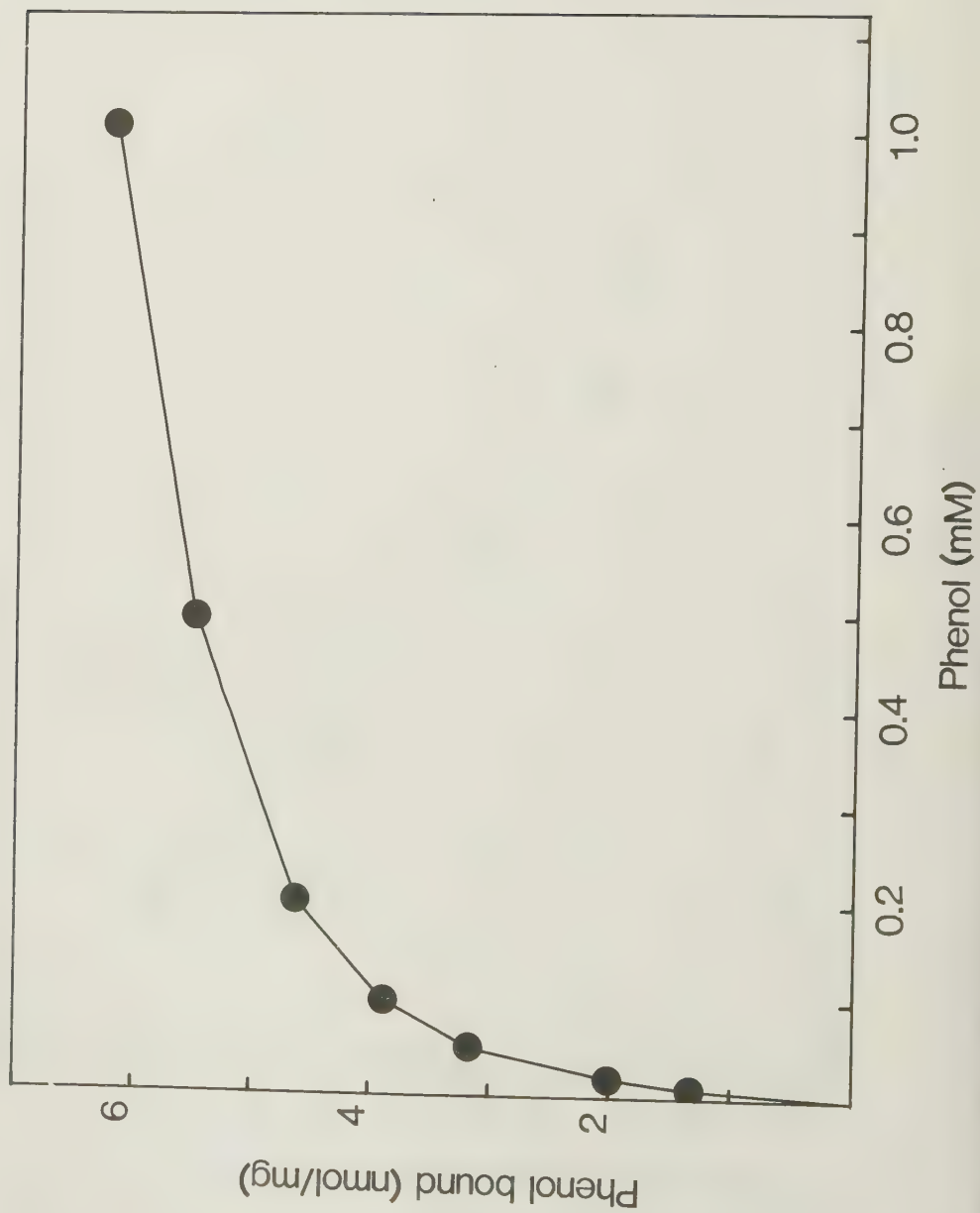


Fig. 3A

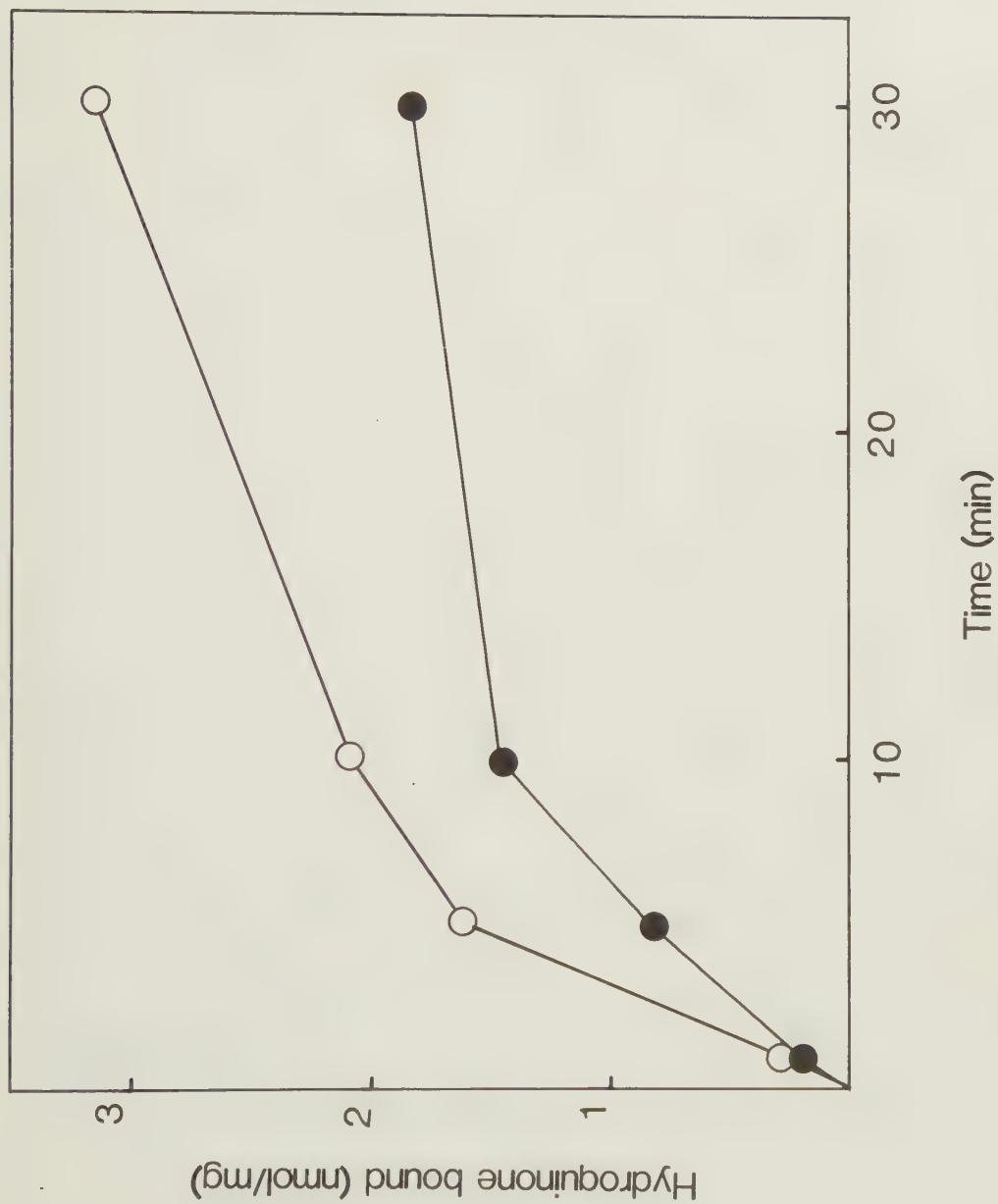


Fig. 3B

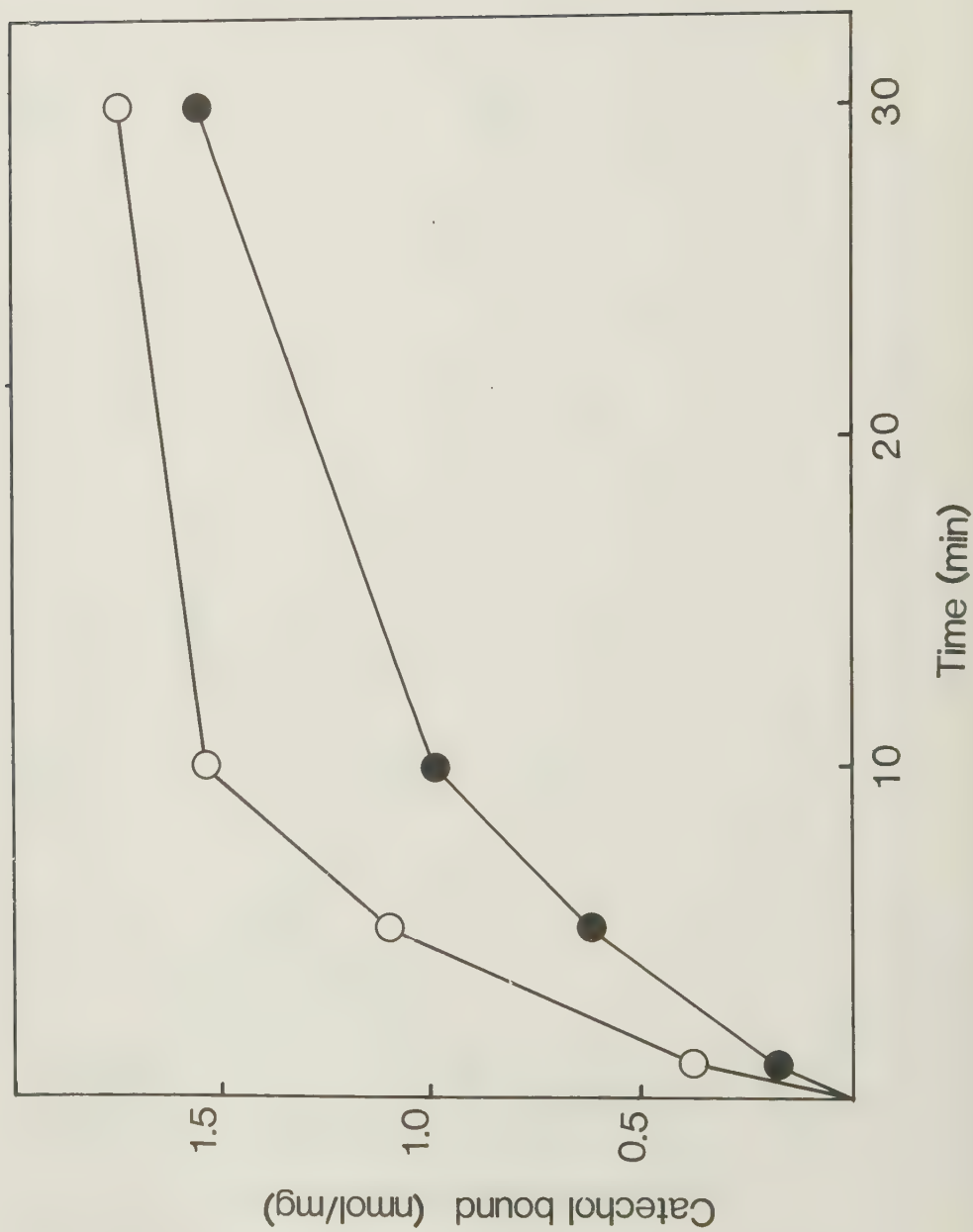


Fig. 4A

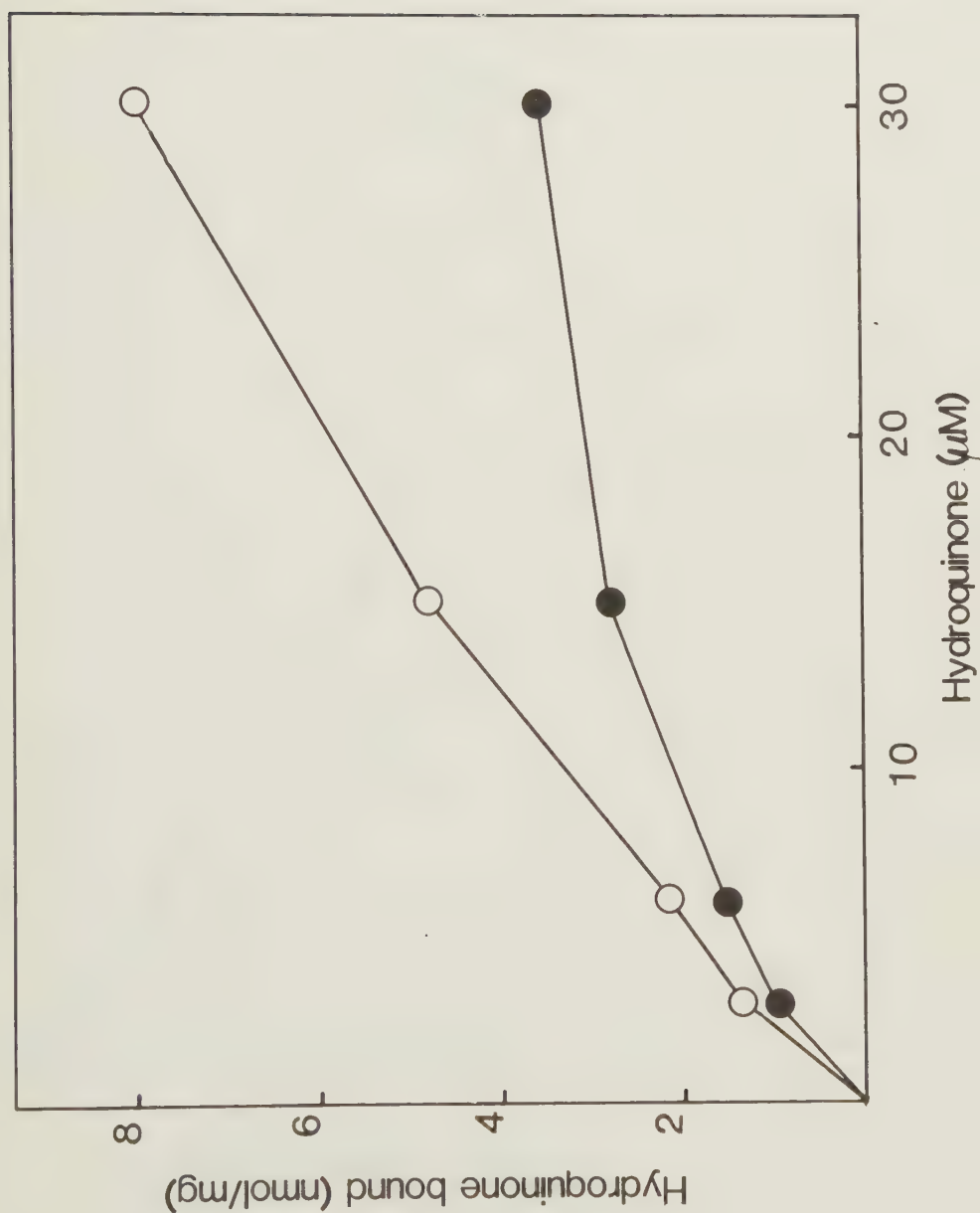
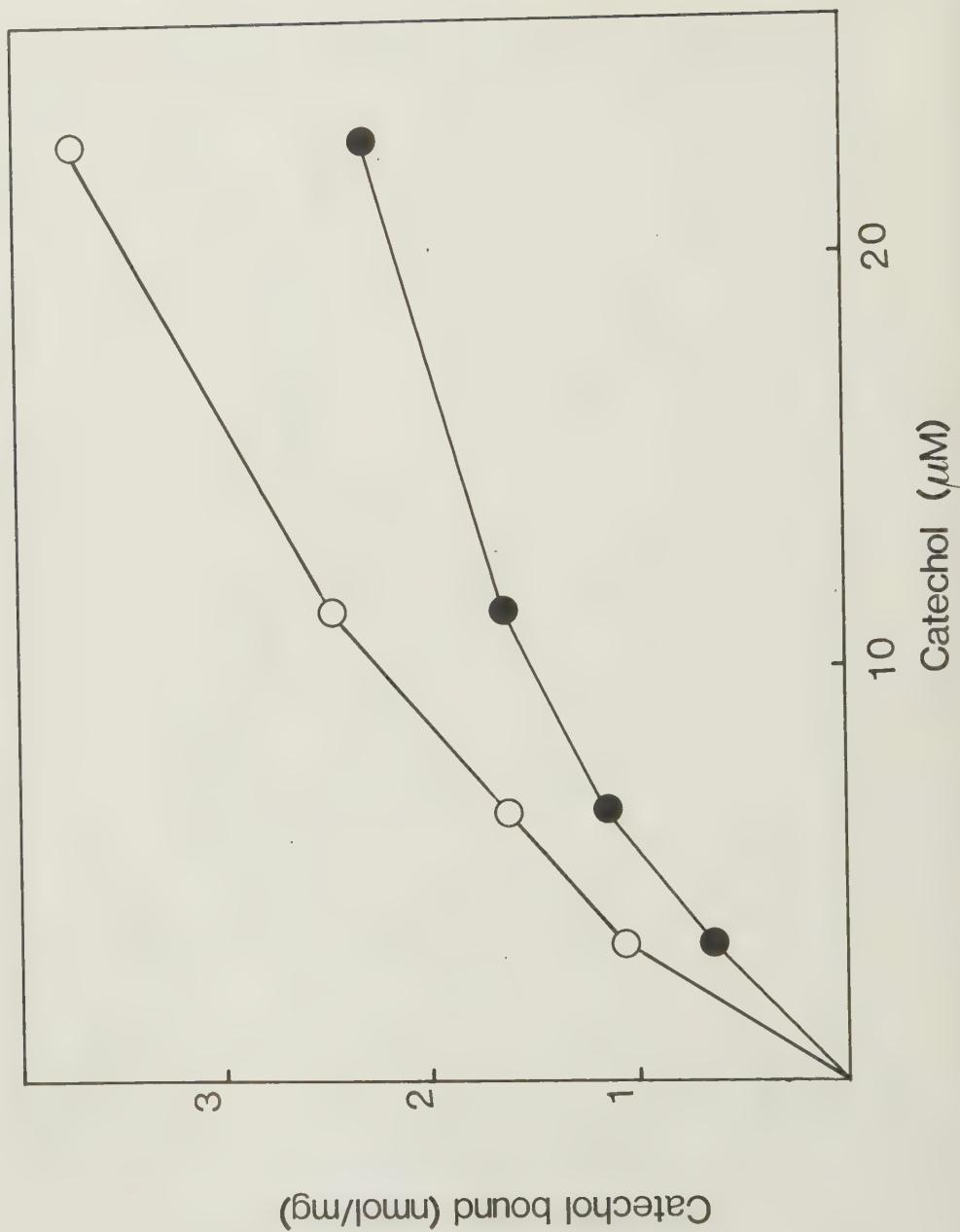


Fig. 4B



IV

Activation of Microsomal Glutathione Transferase Activity during
the Metabolism of Phenol in vitro.

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; SDS, sodium
dodecyl sulphate.

Summary

The activity of microsomal glutathione transferase is increased 1.5-fold in microsomes which carry out NADPH dependent metabolism of phenol. The phenol metabolites benzoquinone and benzenetriol both activate the glutathione transferase in microsomes 2-fold independently of added NADPH. However, NADPH is required to activate the enzyme in the presence of hydroquinone, whereas catechol does not activate the enzyme. Analysis by SDS polyacrylamide gel electrophoresis and fluorography of the proteins that bind reactive metabolites of ^{14}C phenol reveals covalent binding to a protein that comigrates with microsomal glutathione transferase. This indicates that reactive metabolites of phenol activate the enzyme by covalent modification. It is discussed whether the binding and activation has general implications in the regulation of microsomal glutathione transferase and, since some reactive metabolites might be substrates for the enzyme, their elimination through conjugation.

Introduction

Glutathione transferases are a family of detoxifying enzymes with broad and overlapping substrate specificities towards carcinogenic, mutagenic, toxic and pharmacologically active compounds (1,2). Among the substrates are many reactive intermediates formed via the cytochrome P-450 system (1), as well as compounds formed during lipid peroxidation (3).

One member of the glutathione transferase family is membrane-bound and abundant in microsomes (3% of the microsomal protein) (4). This enzyme having similar functional characteristics as the cytosolic transferases, is distinguished by a lower monomer molecular weight and its ability to be activated by sulfhydryl reagents (15-fold by N-ethylmaleimide with purified enzyme) (5). In addition the enzyme is immunologically distinct (see (6) for a review). If the activation by sulfhydryl reagents in vitro reflects a mechanism operating in vivo is presently unknown.

The in vitro metabolism of phenol in rat liver microsomes gives rise to reactive intermediates that bind covalently to proteins (7). Upon SDS-polyacrylamid gel electrophoretic analysis of proteins that bind radioactive phenol metabolites it was found that a labelled component migrated in the region of the microsomal glutathione transferase. This observation led us to investigate whether the microsomal glutathione transferase was indeed a protein that would bind phenol metabolites, and how such binding would influence the activity of the enzyme.

Materials and methods.

All chemicals were of analytical grade. ^{14}C Phenol was from Amersham International. ^{14}C Catechol and ^{14}C hydroquinone were synthesized through enzymatic oxidation of ^{14}C phenol and purified as described (7). Microsomal glutathione transferase (5) and liver microsomes (8) were prepared from male Sprague Dawley

rats (150-200 g, ALAB, Stockholm, Sweden). Protein was determined by the Lowry method (9) using bovine serum albumin as standard.

Activation of microsomal glutathione transferase.

Activation was performed, with small changes, in an assay system used for measuring covalent binding of phenol metabolites to microsomal proteins (7). The reaction mixtures contained 0.5 mg microsomal protein, 100 μ mol NADPH (omitted in controls), 2.5 μ mol MgCl_2 and 25 μ mol Tris-HCl pH, 7.4 in, 500 μ l, and were incubated aerobically at 37°C. Phenol and phenol metabolites were added in concentrations as indicated in each case.

Activation of purified microsomal glutathione transferase was carried out by addition of 5 mM phenol or phenol metabolites to the microsomal glutathione transferase in 10 mM potassium phosphate, pH 7.0, 1 mM glutathione, 0.1 mM EDTA, 20 % glycerol, 1 % Triton X-100 and 0.1 M KCl at room temperature for 1, 5, 10, or 15 min. After incubation aliquots were taken out and the glutathione transferase activity towards CDNB was determined according to Habig and coworkers (10) including 1 % Triton X-100 when the purified enzyme was assayed (8).

Covalent binding of phenol metabolites to microsomal glutathione transferase.

The specificity of the covalent binding of phenol metabolites was investigated by SDS-polyacrylamide gel electrophoresis in 12-20 % slab gels (11). Samples from incubations (see above) containing 50 μ g microsomal protein labelled with ^{14}C phenol, ^{14}C hydroquinone and ^{14}C catechol (all 11 mCi/mmol) were analysed. Fluorography of gels was performed as described (12), with the exception that Amplify (Amersham International) was used as fluorographic amplifier. The location of bound metabolites was also determined by slicing gel lanes into 2 mm slices, that were digested in 200 μ l perchloric acid and 400 μ l 30 % hydrogen peroxide over night

at 60°C. After addition of 5 ml Aquasol (New England Nuclear) radioactivity was determined by liquid scintillation counting. The location of radiolabel was compared with the location of Coomassie Brilliant Blue R-250 stained proteins in a parallel gel lane examined in a 2202 Ultrosan Laser Densitometer (LKB Produkter AB). The microsomal glutathione transferase concentration in some samples was determined immunologically (4).

Results.

Activation of microsomal glutathione transferase activity during metabolism of phenol.

After preincubation of microsomes with phenol and NADPH, the rate of enzymatic conjugation of CDNB with glutathione increased 70 % (Table 1). This increase was dependent on NADPH and on the phenol concentration with a maximum at 100 μ M phenol (Fig. 1a). The maximum activity was reached within 20 s and then slowly declined returning to the control level after 10-20 min (Fig. 1b). Inclusion of 1 mM ascorbic acid or 10 mM glutathione prevented the phenol-dependent increase of the activity. However, if 10 mM glutathione was added to the incubation mixture immediately after the addition of phenol the activity increased as with phenol but did not decline within 10 min (Fig. 1b). Ascorbic acid is believed to prevent oxidation of benzenediols to their corresponding quinones (7,13), and glutathione has been shown to conjugate benzoquinone spontaneously (13).

Activation by metabolites of phenol.

In vivo phenol is transformed to hydroquinone, catechol and 1,2,4-benzenetriol (14). In contrast, only hydroquinone and catechol are formed during microsomal metabolism of phenol in vitro (7,15). It has been suggested that the toxic effects of phenol are exerted by the further oxidation of these metabolites to their corresponding quinones or semiquinones (14). These quinones and semiquinones which can be formed both spontaneously or in enzyme-catalysed reactions (16,17), are electrophilic and capable of binding to cellular macromolecules (13,15). It can be seen in Table 1 that all metabolites except catechol, suggested to be formed during the metabolism of phenol, significantly increase the microsomal glutathione transferase activity towards CDNB. Benzenetriol, benzoquinone and, at 200 μ M or higher concentrations, hydroquinone do not require NADPH to activate

the enzyme, whereas activation by hydroquinone at lower concentrations is dependent on NADPH (Table 1 and Fig. 2a,3a). The changes in activity with time was as for phenol a rapid activation followed by a slow decrease in activity (Fig. 2b 3b).

The effect of phenol and its metabolites on the activity of purified microsomal glutathione transferase was also tested. The concentration of the substances used in the incubations was 5 mM to overcome the inhibitory effect of the 1 mM glutathione present in the buffer. Phenol, hydroquinone and catechol had no effect, while benzoquinone and benzenetriol increased the activity 6- and 3-fold, respectively. Interestingly, hydroquinone at 5 mM did not increase the enzyme activity, whereas 200 μ M increased the activity in microsomes.

Covalent binding of phenol metabolites to microsomal glutathione transferase.

In order to test whether the microsomal glutathione transferase would bind phenol metabolites covalently, microsomes were incubated with radiolabelled phenol, hydroquinone and catechol. Then the microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis and the location of radiolabel was examined by fluorography. In Fig 4, lane b and c it can be seen that a protein of M_r 17 000 bound phenol metabolites to a rather high degree. This protein had an electrophoretic mobility identical with that of purified microsomal glutathione transferase (lane a). Furthermore, when microsomes and purified microsomal glutathione transferase were run together, the radiolabel comigrated with the enzyme. The amount of covalent binding and enzyme in the M_r 17 000 component was determined radiometrically and immunologically respectively. After 10 min of incubation with $200 \mu\text{M}$ ^{14}C phenol the component contained 0.1 mol of bound metabolite (Fig. 5) per mol of glutathione transferase. Radiolabel was also associated with other protein components. The most prominent label migrated in the M_r 50 000 region, probably representing labelling of a cytochrome P-450 isoenzyme(s). Cytochrome P-450 isoenzymes have been found to bind covalently reactive intermediates generated during metabolism of foreign compounds (12,18,19,20,21).

It can be seen in Fig. 4 lane d and e that hydroquinone and catechol became bound to the M_r 17 000 component as well. However, the labelling pattern appears to be less specific than the pattern obtained with phenol.

Discussion.

The activity of microsomal glutathione transferase can be increased in vitro by sulfhydryl reagents, which will modify the cysteine residue of the enzyme (5), or by limited proteolytic

digestion (21). Thus it is possible that the enzyme is regulated in vivo by, for instance, thiol-disulfide exchange , proteolysis or by some other mechanism. Since reactive metabolites of foreign compounds are substrates for glutathione transferases, one attractive idea would be that these metabolites modify the microsomal glutathione transferase covalently, thereby increasing the enzyme activity by which these reactive metabolites are eliminated through conjugation. This would allow the cell to adjust rapidly to exposure to reactive compounds. In comparison adjustment through induction of detoxifying enzymes often takes several days to be fully effected (22). Induction of the microsomal glutathione transferase has not been observed, although the effect of several compounds have been tested (6).

The microsomal metabolism of phenol to species which will bind to proteins is most likely catalyzed by cytochrome P-450 monooxygenases (7,15). About 4 % (after 10 min of incubation) of the phenol metabolites bound to protein, co-migrated with microsomal glutathione transferase on SDS-polyacrylamide gel electrophoresis (Fig. 5). The preferential labelling of glutathione transferase suggests either that the reactive metabolites have a higher affinity for this protein than for most other proteins present in microsomes (which would be expected since similar compounds are substrates for the enzyme), or that the transferase is juxtaposed to cytochrome P-450 in the microsomal membrane thereby facilitating the binding of reactive metabolites. Interestingly, cytosolic glutathione transferases have been shown to be targets for binding of reactive metabolites. Thus, the two subunits of glutathione transferase B were preferentially labelled during microsomal metabolism of benzo(a)pyrene in the presence of cytosolic proteins (24,25).

The increase of microsomal glutathione transferase activity during phenol metabolism is very rapid but the activity soon decreases (Fig. 1b). Presumably, the increase in activity is due to the covalent binding of metabolites to the enzyme. The reason

for the following decrease is not known presently. However, the enzyme is very labile, and does not tolerate solubilisation or storage without glutathione (unpublished observations). Since glutathione prevented the decline in activity it is possible that electrophilic metabolites affect the enzyme or other membrane components through, for instance, oxidative stress.

A further finding was that all metabolites generated from phenol, except catechol, significantly increased glutathione transferase activity in microsomes. It is possible that the NADPH dependent activation of the enzyme by hydroquinone is caused by benzoquinone formed through enzymatic metabolism of hydroquinone (17), and that the NADPH independent activation at higher concentrations (Fig. 2a) is caused by autooxidation of hydroquinone. This is consistent with the activation of purified glutathione transferase by benzenetriol and benzoquinone. The inability of hydroquinone to activate the enzyme could be explained by a rapid elimination of benzoquinone formed through spontaneous conjugation with glutathione. It has been found that benzenetriol autooxidizes faster than hydroquinone (16).

The mechanism of activation of microsomal glutathione transferase by metabolites of phenol is probably analogous to the effect of sulfhydryl reagents. It is likely that the electrophilic metabolites benzoquinone and 2-hydroxybenzoquinone conjugate with the sulfhydryl group of the enzyme, thereby activating the enzyme. Interestingly, phenol, hydroquinone and catechol cause similar toxicity and cellular effects as sulfhydryl reagents (26). Irons and Neptun (27) found that hydroquinone inhibited the polymerisation of microtubuli in vitro which also sulfhydryl reagents do, and that hydroquinone became irreversibly bound to the high molecular weight fraction of tubulin.

In order to evaluate the physiological significance of our in vitro observations, we have to find out if any of the metabolites of phenol are substrates for the microsomal glutathione

transferase and that the enzyme activity is increased towards these compounds. It has earlier been found that the activity towards certain substrates is increased by modification of the enzyme, while the activity towards other substrates remains unchanged (5). The results presented here agree with those of Botti et al. (28), who found that treating rats with 1,2-bromoethane or CCl_4 increased the microsomal glutathione transferase activity by up to 50 % within 2 hours. Both these substances are metabolically activated and bind to proteins (29,30).

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Legends to figures.

Fig. 1a. Changes in glutathione transferase activity after incubation with phenol. Closed circles are incubations with and open without NADPH.

Fig 1b. Activity after incubation for different times with 200 μ M phenol closed circles phenol only; squares, glutathione was added immediatly after phenol.

Fig. 2a. Glutathione transferase activity in microsomes after preincubation with different concentrations of hydroquinone for 20 s. Closed circles are preincubations with and open circles without NADPH.

Fig. 2b. Glutathione transferase activity in microsomes after preincubation with(closed circles) and without(open circles) 25 μ M hydroquinone (closed circles) or 1,2,4-benzenetriol (squares) for different times as indicated in the figure.

Fig. 3a. Glutathione transferase activity after preincubation of microsomes for 20 s with 25 μ M benzoquinone in the presence (closed circles) or absence (open circles) of NADPH.

Fig. 3b. Glutathione transferase activity in microsomes after preincubation with benzoquinone for different times. The concentrations of benzoquinone used are shown in the figure.

Fig. 4. Electrophoretogram of microsomal proteins and protein binding patterns of phenol metabolites. a, purified microsomal glutathione transferase; b, rat liver microsomes stained with Coomassie Brilliant Blue R 250; fluorography of proteins labelled during incubation, as described in Materials and methods with 200 μ M 14 C phenol (c), 30 μ M 14 C Catechol (d) or 30 μ M 14 C hydroquinone (e) , The molecular weight markers used were, horse heart cytochrome c, sperm whale myoglobin, soy-bean trypsin

inhibitor, ovalbumin and bovine serum albumin. The molecular weight of M_r the microsomal glutathione transferase in another electroporesis system was reported reported to be M_r 14 000 (8). The M_r obtained here was 17 000 which agrees well with the molecular weight computed from the primary sequence of the enzyme.

Fig. 5. Separation of microsomal proteins by electrophoresis. Microsomes were incubated for 10 min with 200 μ M 14 -phenol. After electrophoresis gels were either sliced and analysed for radioactivity (A) or stained for protein and scanned. The positions of M_r markers are indicated at bottom.

Treatment	+NADPH		-NADPH	
	Specific activity (nmol/mg min)	Relative activity	Specific activity (nmol/mg min)	Relative activity
none	85 $\pm$ 6	1.00	91 $\pm$ 4	1.00
200 μ M Phenol	142 ^a $\pm$ 10	1.67	107 $\pm$ 9	1.18
25 μ M Hydroquinone	180 ^a $\pm$ 13	2.12	108 $\pm$ 10	1.19
25 μ M Benzoquinone	147 ^a $\pm$ 8	1.73	192 ^a $\pm$ 8	2.11
25 μ M Benzenetriol	201 ^a $\pm$ 12	2.36	198 ^a $\pm$ 7	2.18
25 μ M Catechol	102 $\pm$ 6	1.20	98 $\pm$ 7	1.08

Table 1. Specific and relative activity of microsomal glutathione transferase after treatment with phenol and phenol-metabolites. Measurements were performed as described under methods. The relative activity is the ratio between the specific activities of treated and untreated microsomes. The standard deviation of the mean (n=6) are given for the specific activities.

a. Significantly different from control values (student t-test, $F(x)=0.99$).

Fig. 1A

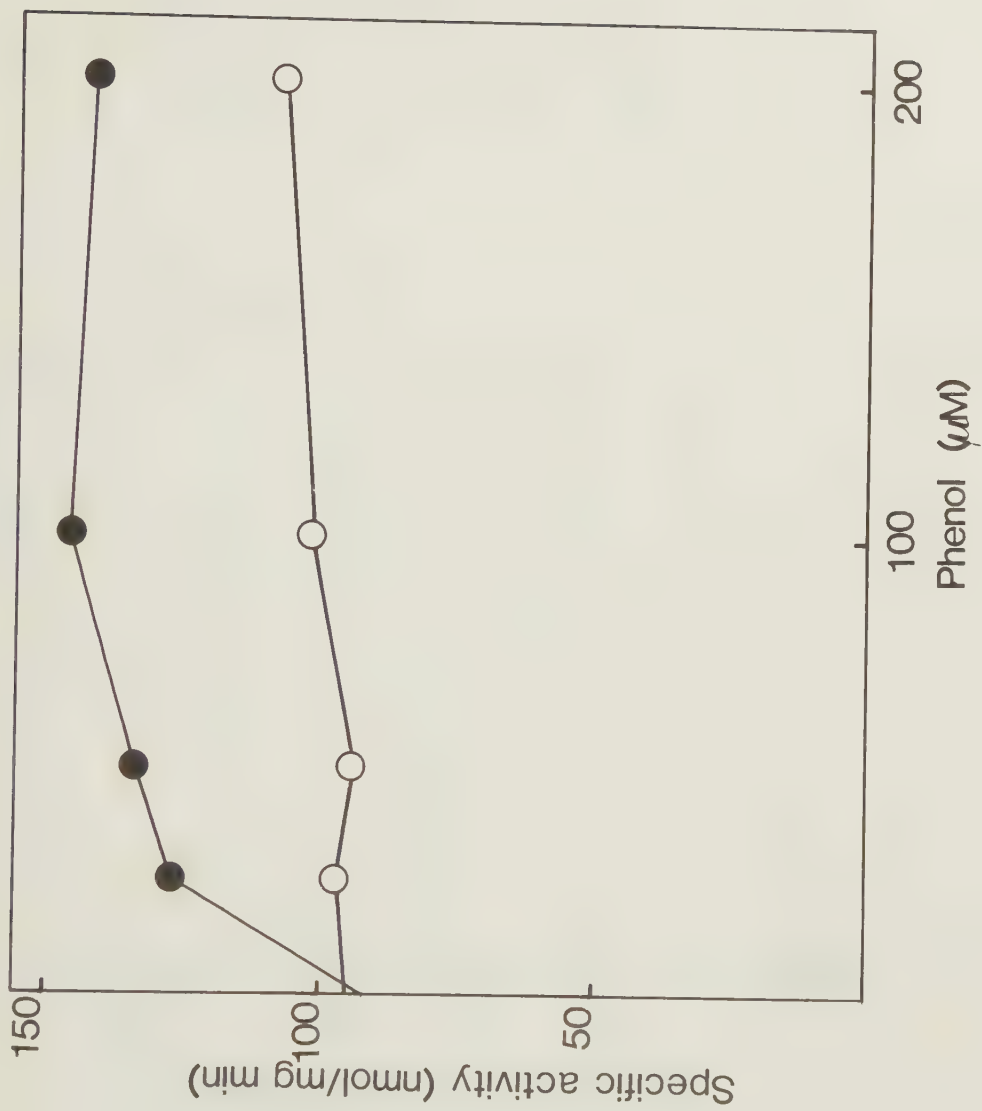


Fig. 1B

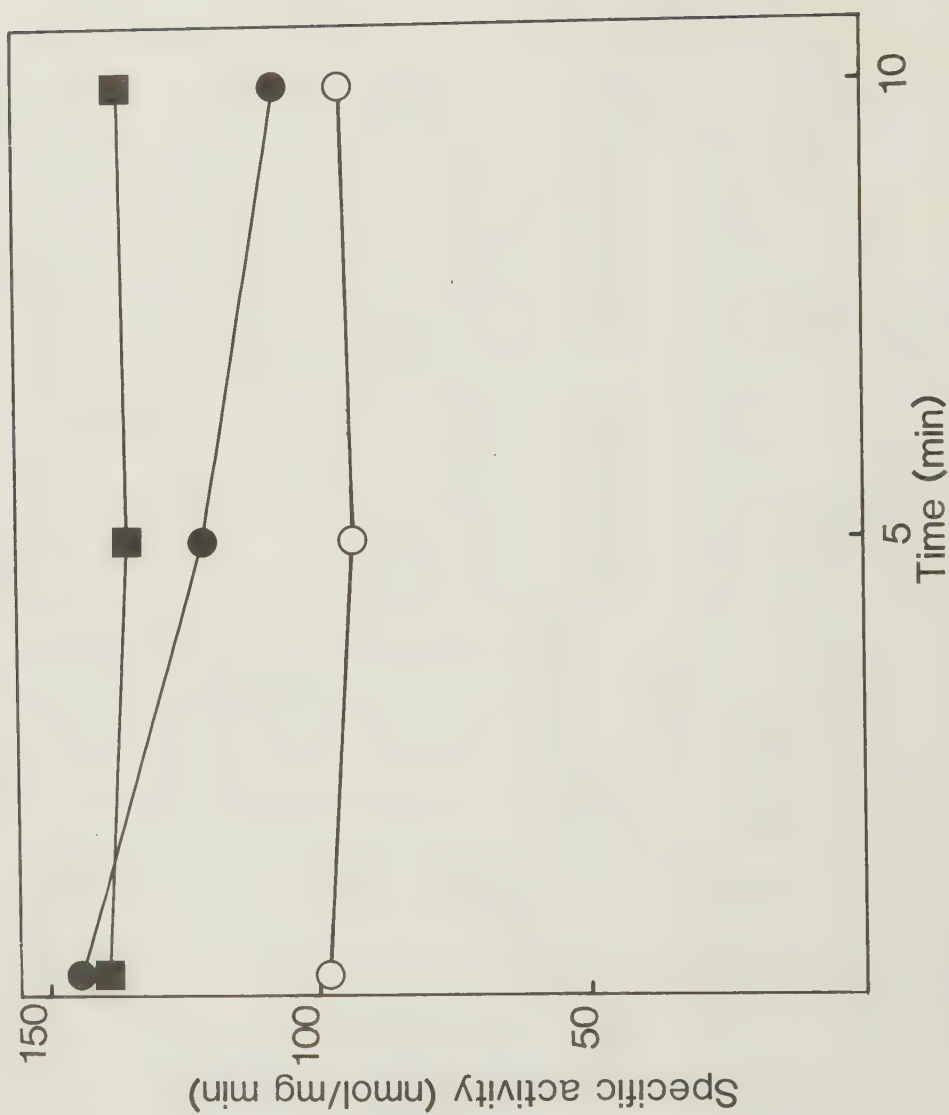


Fig. 2A

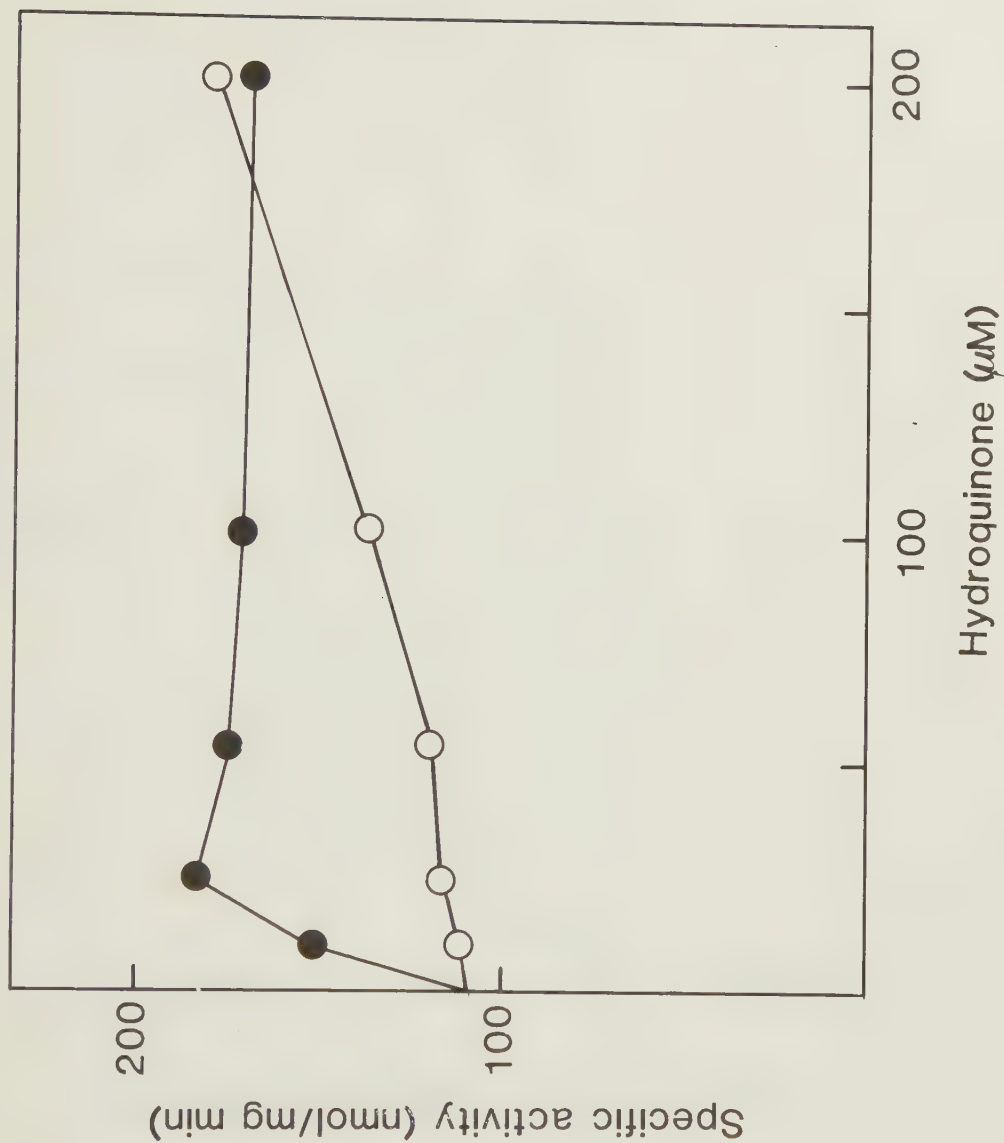


Fig. 2B

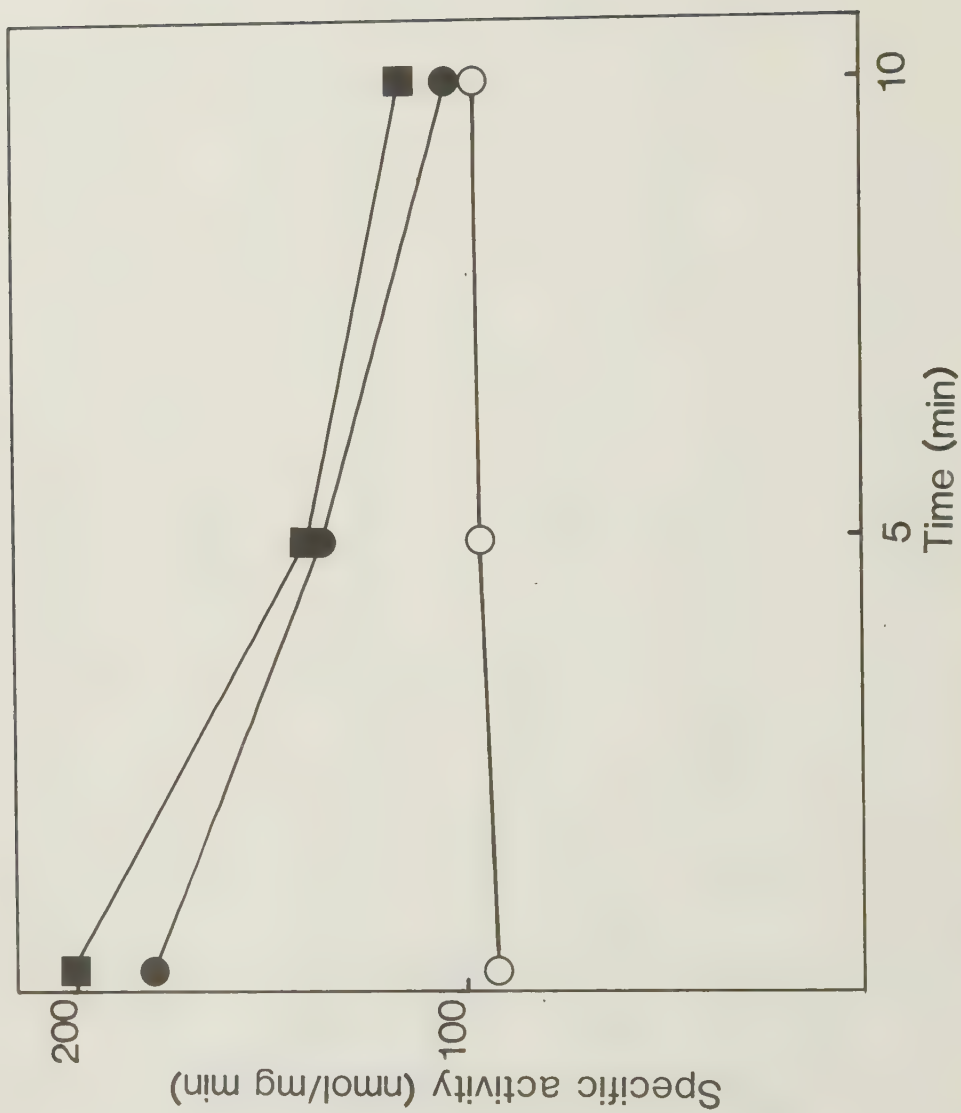


Fig. 3A

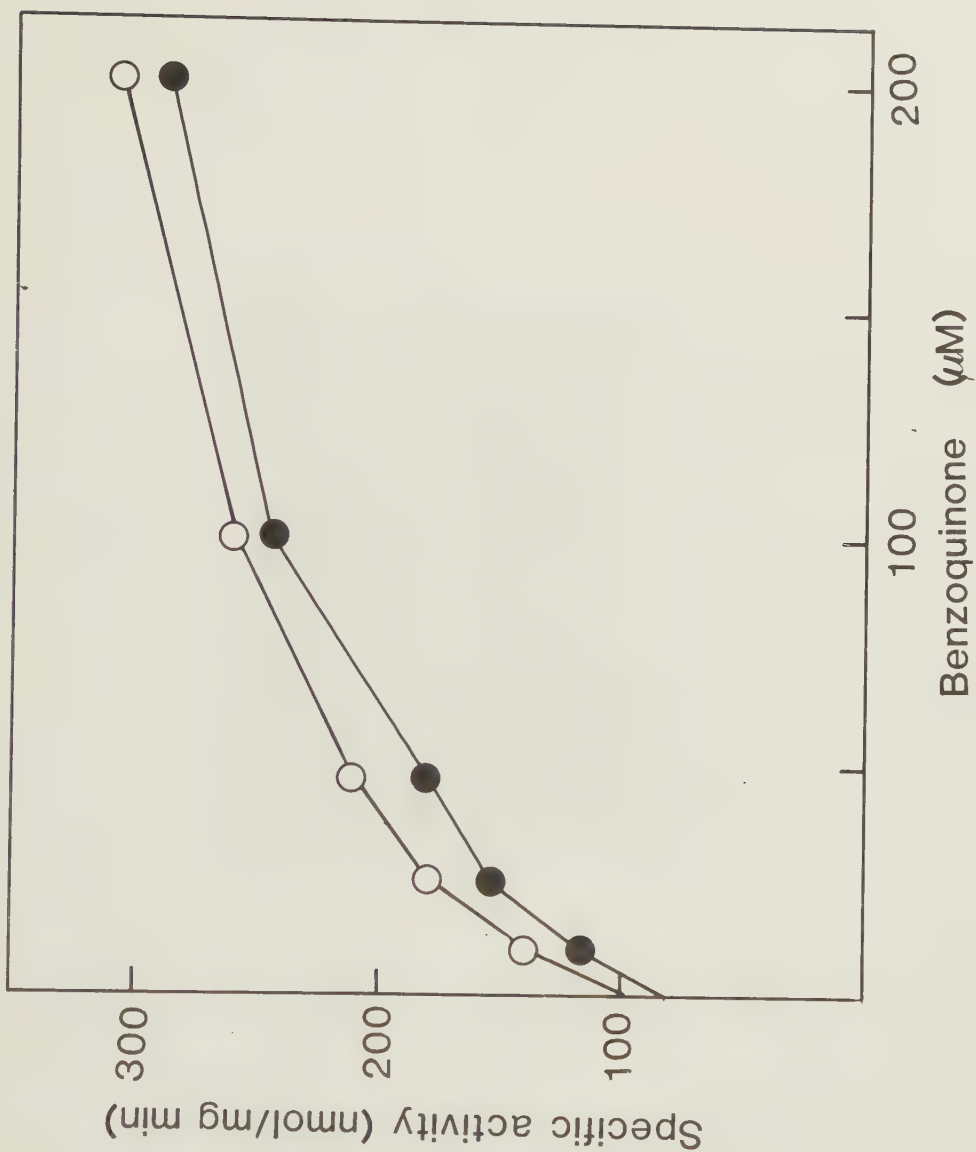


Fig.3B

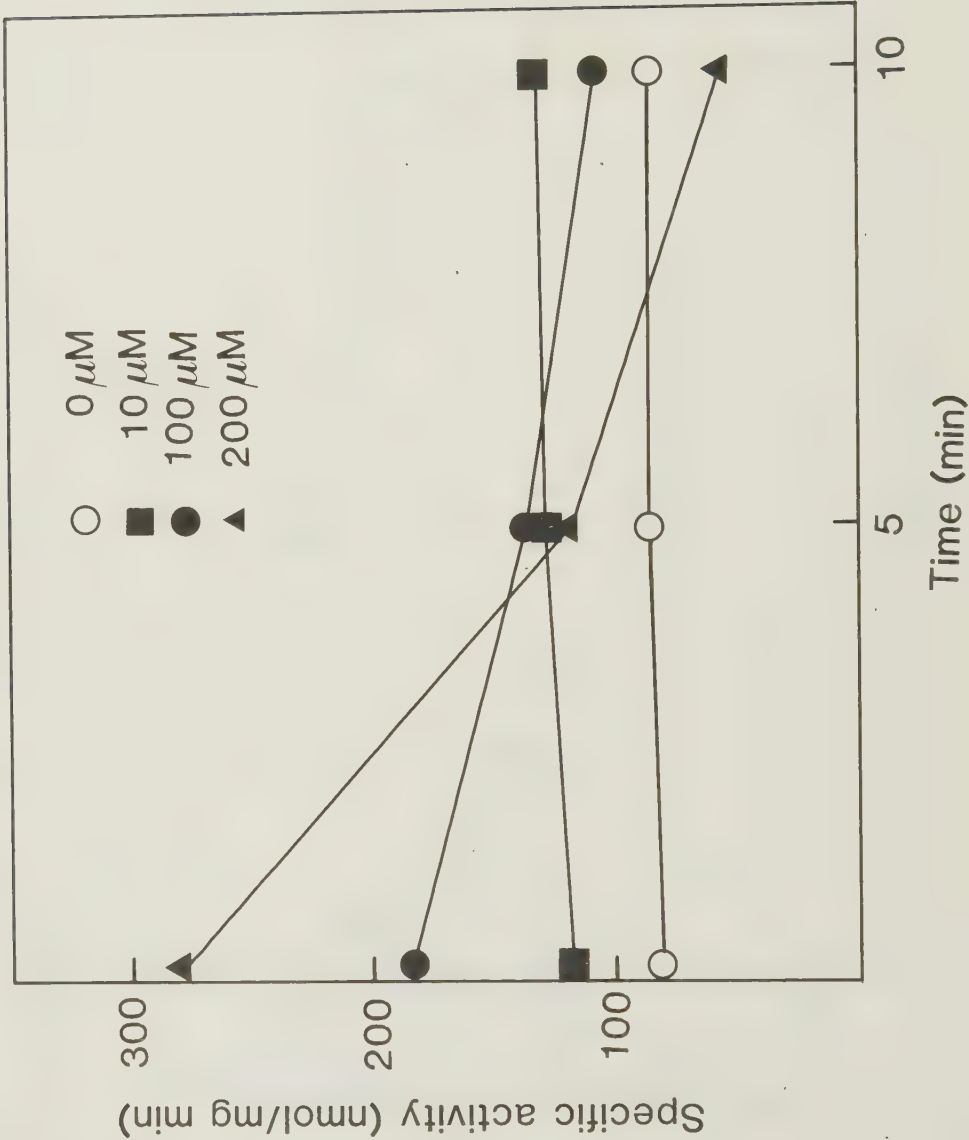


Fig. 4.

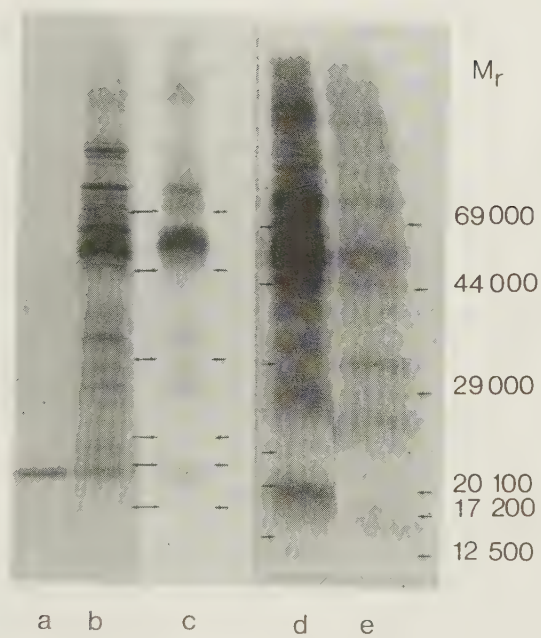
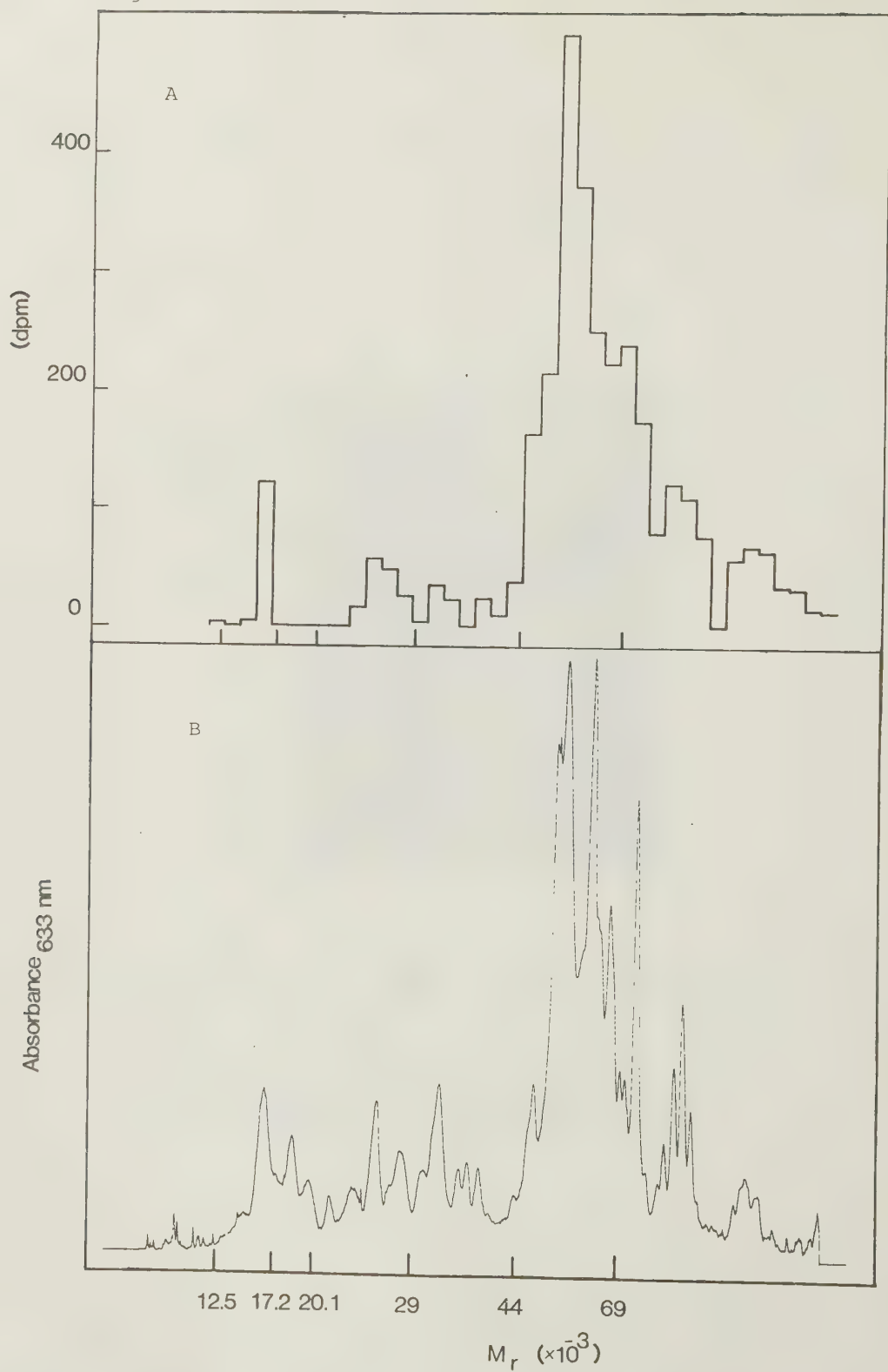


Fig.5



v

ENZYME IMMUNOASSAY OF BENZO[*a*]PYRENE CONJUGATED TO DNA, RNA AND MICROSOMAL PROTEINS USING A MONOCLONAL ANTIBODY

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SUMMARY

An enzyme immunoassay for the detection of benzo[*a*]pyrene covalently conjugated to macromolecules has been developed. The monoclonal antibody, raised through *in vitro* immunization reacted with benzo[*a*]pyrene metabolites bound to DNA, RNA and proteins. The lower detection limit for the assay was 1 pmol for benzo[*a*]pyrene bound to DNA or RNA, and 5 pmol when bound to protein.

INTRODUCTION

Polyaromatic hydrocarbons (PAH) are widespread pollutants in industrial and urban environments. They occur where fossil fuels are incompletely combusted, where tar and asphalt are handled, in smoked food and in tobacco smoke. Exposure to PAH is related to the development of cancer in humans [6]. PAH are metabolized by microsomal enzymes to reactive intermediates which will bind covalently to nucleophilic sites in macromolecules. Binding to DNA can result in mutations which may ultimately lead to the development of cancer [14]. Benzo[*a*]pyrene is one of the most studied PAHs and the development of methods to monitor benzo[*a*]pyrene bound to macromolecules is of great interest.

Immunological techniques are advantageous for measuring substances in low concentration in biological materials. Such techniques have been used to detect carcinogens and their adducts with cellular macromolecules [12].

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Immunological methods for the detection of benzo[a]pyrene bound to DNA have been reported [15,18]. The use of monoclonal antibodies in these methods would be advantageous since they can be produced in large amounts and thus used as standard reagent in several laboratories, for instance in investigations of the mechanism of chemical carcinogenesis.

In this paper we report the development of an enzyme linked immunosorbent assay using a monoclonal antibody for the detection of benzo[a]pyrene bound to DNA, RNA and proteins.

MATERIALS AND METHODS

Antigens

6-Amino-benzo[a]pyrene was synthesized according to Creech [3] and was coupled to bovine serum albumin as follows. BSA (20 mg) was dissolved in 2 ml of 10 mM Tris-HCl (pH 8.8), before addition of 10 mg of 6-amino-benzo[a]pyrene in 0.7 ml dioxane. The reaction was started by addition of 100 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma Chemical Co., MO, U.S.A.) and run at pH 8.0 for 1 h. Unreacted 6-amino-benzo[a]pyrene was precipitated by addition of 10 ml of water and the haptenized protein-antigen was further purified by passing it over a column (10 × 40 mm) containing Amberlite XAD-2. After concentration on an Amicon Macrosolute Concentrator the solution was passed over a Pharmacia PD-10 column. The macromolecular fraction was lyophilized, and the degree of substitution was determined spectrophotometrically to 7 haptenes per molecule of bovine serum albumin. Benzo[a]pyrene was also coupled to ovalbumin by generation of a benzo[a]pyrene carbonium ion which reacts spontaneously with nucleophilic centers in proteins. Coupling was accomplished by dissolving 20 mg of benzo[a]pyrene and 95 mg iodine in 25 ml ethanol before mixing with 50 mg ovalbumin in 45 ml of 0.1 M sodium phosphate buffer (pH 6.8). The solution was stirred in the dark for 2 h, before purifying the antigen by extractions in organic solvents according to Tunek and co-workers [19]. The number of benzo[a]pyrene residues linked to ovalbumin was determined radiometrically to 16 per ovalbumin molecule.

Benzo[a]pyrene was also activated to reactive metabolites by incubation with rat liver microsomes. Reactive metabolites formed will then bind covalently to microsomal proteins. Incubation was performed for 7 h as described in Ref. 21, except that benzo[a]pyrene was added in 10 μ l dimethylsulfoxide to a total of 6 ml incubation mixture. The macromolecular fraction was purified as described in Ref. 19. Conjugation of benzo[a]pyrene to DNA and RNA was achieved enzymatically as follows. Five milligrams of calf thymus DNA or of yeast RNA (Sigma, fraction XI) were incubated with 5 mg of liver microsomal protein from 3-methylcholanthrene pretreated rats, 400 nmol (DNA) or 125 nmol (RNA) 14 C-labelled benzo[a]pyrene, 10 μ mol NADPH, 10 μ mol MgCl_2 , 250 μ mol Tris-HCl (pH 7.4),

in a total volume of 5 ml for 1 h (DNA) or 30 min (RNA) at 37°C. DNA was then isolated essentially as described in Ref. 9 and RNA as in Ref. 18. The purified DNA contained 170 pmol and the RNA 40 pmol of benzo[a]-pyrene conjugated per mg nucleic acid.

Monoclonal antibody

A monoclonal antibody against benzo[a]pyrene was produced by utilizing an in vitro immunization technique recently described by Borrebaeck [1]. Briefly, $4-6 \times 10^6$ spleen cells/ml from non-immunized BALB/c mice were cultured in the presence of 50 μ g benzo[a]pyrene (conjugated to bovine serum albumin) per ml medium. The culture medium used in the in vitro immunization was also supplemented with a supernatant from a mixed thymocyte culture as a source of allogeneic lymphokines. After 5 days of immunization in culture the spleen cells were immortalized by fusing them with Sp2/0-Ag14 [15] as previously described [1]. Several clones producing monoclonal antibodies against benzo[a]pyrene could be isolated after the hybrid cells had been cloned twice. This investigation was performed using the 3A1-B2 antibody.

Immunodiffusion

Immunodiffusion was performed according to the method of Ouchterlony [13] using 0.8% agarose and 0.075 M sodium barbital buffer (pH 8.6).

Enzyme immunoassay

One hundred microliters of benzo[a]pyrene-protein, DNA, or RNA conjugate was added to each of 96 wells of a microtiter plate (Dynatech Micro ELISA system M1298) and dried overnight at 37°C. After the plate had been coated with the benzo[a]pyrene conjugate it was washed 3 times in 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.05% Tween 20 (PBS-Tween). One hundred microliters of a monoclonal antibody (3A1-B2) against benzo[a]pyrene, diluted in 10 mM phosphate buffer (pH 8.0), also containing 0.5 mM NaCl and 0.1% Tween 20, was added to all wells and incubated for 30 min at 37°C. The plate was washed 5 times in PBS-Tween before addition of 100 μ l rabbit antimouse Ig conjugated to horseradish peroxidase (Dako Patt A/S, Denmark) diluted 1:400 in 10 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl and 0.1% Tween 20, and incubation for 30 min at 37°C. Unbound conjugate was finally removed by rinsing the plate 5 times as in the previous steps before addition of 100 μ l enzyme substrate (0.4 mM 2,2'-azino di-(3-ethylbenz-thiazoline sulfonic acid) diammonium salt and 0.3% H₂O₂ in 0.1 M sodium citrate buffer, pH 4.0). The plate was analyzed spectrophotometrically at 405 nm after 30–60 min, using a Flow Multiskan photometer.

RESULTS AND DISCUSSION

To test the specificity of the monoclonal antibody we used benzo[a]-

pyrene conjugated to ovalbumin instead of bovine serum albumin which had been used in the *in vitro* immunization step. Microtiter plates pre-coated with benzo[a]pyrene conjugated to ovalbumin were used in the enzyme immunoassay. Figure 1 shows the dose-response relation obtained using antibodies diluted 1:10–1:2000. Fifty percent of maximum binding was recorded at a 1:500 dilution of the monoclonal antibody. Double immunodiffusion was, furthermore, used to test the specificity of the anti-benzo[a]pyrene antibodies. A specific line of precipitation was obtained between benzo[a]pyrene conjugated to ovalbumin and the monoclonal antibodies, whereas no precipitation lines could be detected when unconjugated ovalbumin was tested. As a further control, several monoclonal antibodies with unrelated specificities did not form any precipitates with the benzo[a]pyrene-ovalbumin conjugate. These tests reveal that the 3A1-B2 antibody was directed against the benzo[a]pyrene molecule, and not against the carrier protein or the chemical linkage between benzo[a]pyrene and the protein used in the immunization step.

The monoclonal antibody was then used to determine the amount of benzo[a]pyrene bound to microsomal proteins. Benzo[a]pyrene was activated by incubation with rat liver microsomes. Reactive metabolites formed then bound covalently to microsomal proteins. A preparation containing 8.6 nmol of benzo[a]pyrene/mg protein was used in the test. Microtiter plates were coated with various amounts of the microsomal protein preparation to examine the dose-response relation as obtained by the enzyme

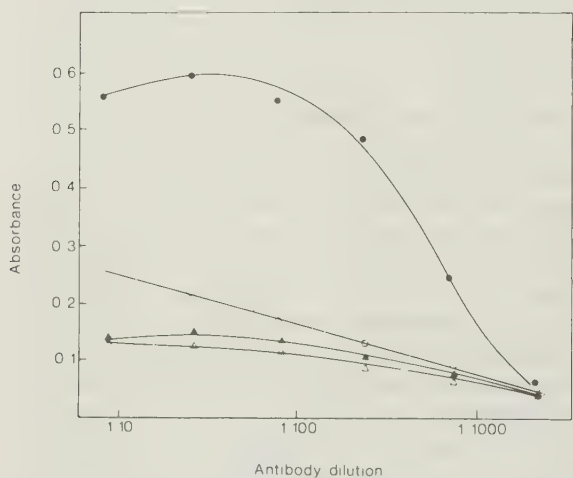


Fig. 1. Dose-response relation of a monoclonal antibody against benzo(a)pyrene as determined by the enzyme immunoassay. An anti-benzo(a)pyrene antibody was tested in microtiter plates pre-coated with benzo(a)pyrene-ovalbumin (●—●) or with native ovalbumin (○—○). A monoclonal antibody exhibiting an unrelated specificity was also tested as a control against benzo[a]pyrene-ovalbumin (▲—▲) or native ovalbumin (△—△).

immunoassay (Fig. 2A). The lower detection limit was 5 pmol of benzo[a]-pyrene bound to microsomal proteins.

The amount of benzo[a]pyrene enzymatically bound to DNA or RNA was also quantitated in a similar manner as described for the microsomal proteins. Figure 2B shows the result of an enzyme immunoassay where a serial dilution of benzo[a]pyrene-DNA conjugates had been used to coat the wells of microtiter plates. The monoclonal antibody showed a strong reactivity to the conjugate between benzo[a]pyrene and DNA, whereas native DNA showed no reactivity at all. Figure 2C shows the result of a similar enzyme immunoassay where benzo[a]pyrene was conjugated to RNA. The detection limit for benzo[a]pyrene bound to DNA or RNA was approximately 1 pmol, respectively. The higher sensitivity for detecting benzo[a]pyrene bound to DNA or RNA than to microsomal proteins was most probably due to the accessibility of the hapten molecule to the antibody. In microsomes benzo[a]pyrene is suspected to be bound in hydrophobic regions of the membrane structure resulting in less accessibility for the monoclonal antibody. The detection limit using our monoclonal antibody was comparable to what has been reported for an assay of aflatoxin B₁ adducts in DNA using monoclonal antibodies [5].

Development of the in vitro immunization methodology has made it possible to specifically immunize mouse spleen cells and to use these cells in somatic cell hybridization experiments [1,2,10,11] for production of antigen-specific monoclonal antibodies. This technique has also been useful in our case when monoclonal antibodies against a very weak immunogen were to be produced, since the regulation of the in vivo immune response seems to be suppressed in culture systems.

The enzymatic procedure used for conjugation of benzo[a]pyrene to DNA, RNA and proteins results in the binding of several different benzo[a]-pyrene metabolites to various target sites in the macromolecules. Jernström et al. [8] have shown that metabolites of 9-hydroxybenzo[a]pyrene and 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene (presumably the 4,5- and 9,10-oxides, respectively) are the major species that will bind to DNA under similar conditions as those used here. There were also indications for different target sites in DNA [8], whereas the binding to microsomal proteins is less well characterized. We have shown, however, that metabolites of 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene will bind more efficiently than other metabolites tested [16], and that several microsomal proteins are targets for metabolically activated benzo[a]pyrene [19]. Details of the binding of benzo[a]pyrene to RNA are not known.

Since the monoclonal antibody, raised against a benzo[a]pyrene conjugated to albumin, reacted with the hapten bound both to DNA, RNA, microsomal proteins and ovalbumin, the site of binding in the macromolecule appears less critical for antibody recognition. This is in contrast to an earlier report where the specificity of an anti-benzo[a]pyrene antibody depended on how the hapten was bound to a macromolecular site, and only reacted with benzo[a]pyrene bound to DNA [7].

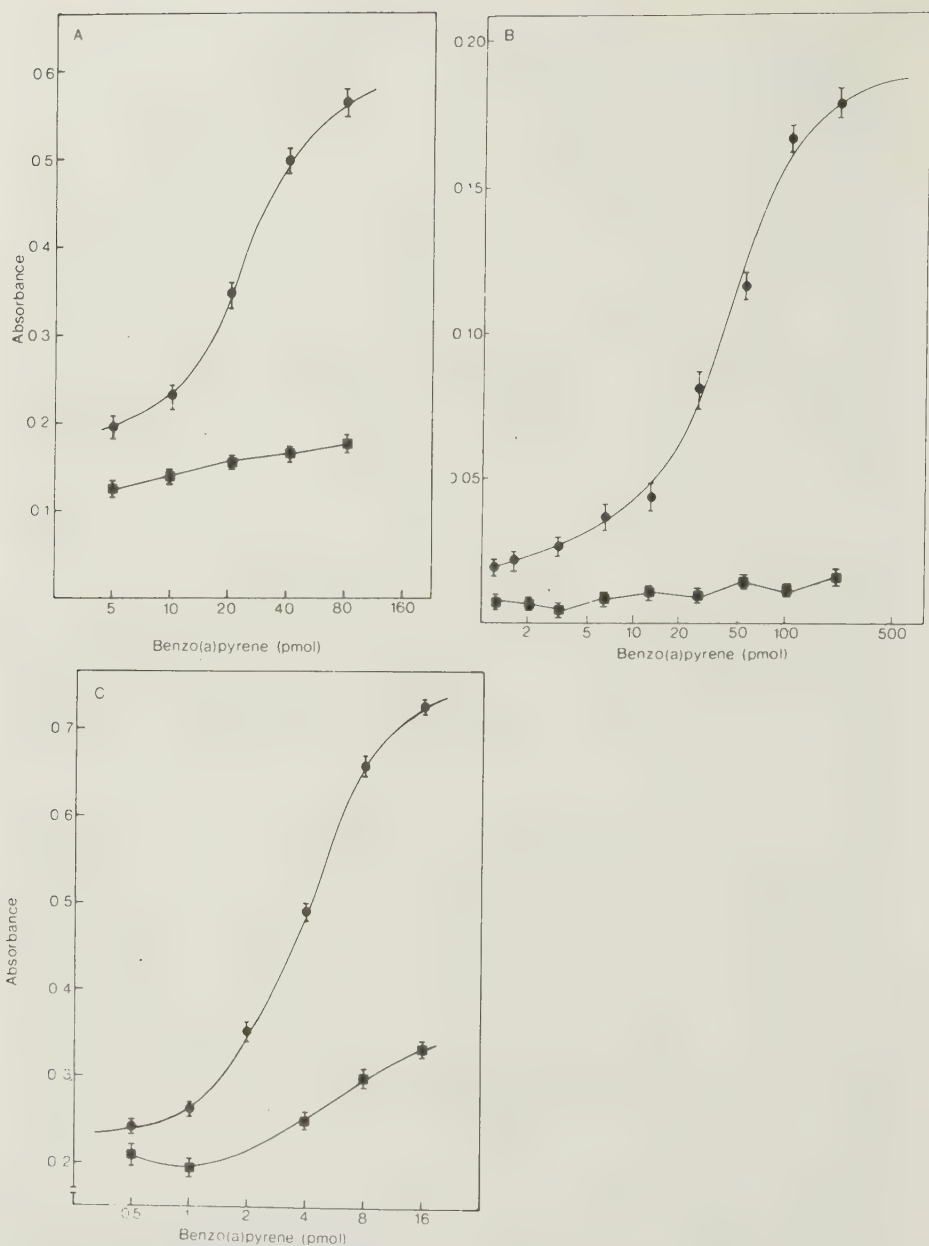


Fig. 2. Enzyme immunoassays (●—●) of serially diluted conjugates between benzo-*[a]*pyrene and (A) microsomal proteins (8.6 nmol benzo-*[a]*pyrene/mg protein); (B) DNA; (C) RNA. The coefficient of variation in the immunoassays was 5% in A and B, and 7% in C. The negative control values (■—■) were obtained by omitting the specific antibody in the enzyme immunoassay.

The fact that the monoclonal antibody developed against benzo[a]pyrene coupled via an amido link in the 6-position reacts with benzo[a]pyrene metabolized and conjugated to macromolecules in other positions indicates that the specificity of the antibody is directed against ring structures distal to sites of conjugation.

The application of the monoclonal antibody for detection and quantitation of macromolecular adducts resulting from environmental exposure to benzo[a]pyrene will most probably require a more sensitive immunoassay. It would, however, be possible to increase the sensitivity by using enzymatic radioimmunoassay [7] based on radioactive substrates, or high-sensitive enzyme-linked immunosorbent assay [20] based on fluorescent products.

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